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Mode of action of ammonia and amine on rRNA synthesis in *Xenopus laevis* embryonic cells

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Summary

Recently, we found that rRNA synthesis in dissociated embryonic cells of *Xenopus laevis* is selectively inhibited by ammonium salts added to their surrounding medium. Similar effects were also observed in cells treated with amines. In this report, we analysed in more detail the effects of an ammonium salt and trimethylamine on rRNA synthesis in *Xenopus* embryonic cells cultured under several different conditions.

Results obtained showed that ammonia and trimethylamine inhibit formation of 40S pre-rRNA, without inducing breakdown (wastage) or aberrant processing of the 40S pre-rRNA. Such inibitory effects were also shown not to be due to interference with cellular ATP level. Furthermore, we found that the effects of both ammonium salt and trimethylamine were completely abolished when Na^+ in the culture medium of the testing cells was replaced by choline ions. These results favour the view that the inhibition of rRNA synthesis by ammonia and amine is realized at the transcriptional level or at the step before the formation of 40S pre-rRNA, most probably *via* a slight increase of intracellular pH, but not *via* an inhibition in energy-generating systems.

Key words: ammonia, amine, weak base, rDNA transcription, pH effect.

Introduction

It has been well established that the synthesis of rRNA is closely related to, and hence sensitively reflects, cellular physiological state (e.g. Lieberman, Abrams & Ove, 1963; Cooper, 1968; Misumi, Nishio, Shiokawa & Yamana, 1978). In the development of *Xenopus laevis* fertilized eggs, which are unique cells that contain ribosomes the bulk of which are used in later development, rRNA synthesis is regulated in such a way that commences at the late blastula stage (Shiokawa *et al.* 1981*a*,*b*), just at, or shortly after, the timing which is now called MBT (midblastula transition) (Newport & Kirschner, 1982). Since developing *Xenopus* embryos need newly synthesized ribosomes for their survival only at the swimming tadpole stage (Brown & Gurdon, 1964), the onset of rRNA synthesis at as early as the late blastula stage may be an extremely safe device to ensure the supply of ribosomes.

As for the mechanism of such a 'developmental' regulation of rRNA gene expression, the classical nuclear transplantation experiment by Gurdon & Brown (1965) strongly suggested the involvement of a cytoplasmic controlling factor. However, the molecular mechanism of the onset of the rDNA expression at this particular developmental stage (the late blastula stage) has not yet been clarified, although several mechanisms such as the involvement of transcription stimulators (Crampton & Woodland, 1979; Russell, 1983), changes in rDNA chromatin configuration (Reeves, 1978; Spadafora & Riccardi, 1985) and rDNA methylation (Bird, Taggart & Macleod, 1981) have been proposed.

In our studies of the regulative mechanism of rDNA expression in *Xenopus* embryogenesis, we have recently found that various ammonium salts and amines added at the concentration of about 3 mM in the surrounding medium selectively inhibit rRNA synthesis in *Xenopus* neurula cells (Shiokawa, Kawazoe & Yamana, 1985; Shiokawa *et al.* 1986*a*,*b*). Furthermore, quite unexpectedly, we found that ammonia exists in unfertilized eggs and embryos at an intraegg concentration of about 3 mM and it decreases

two- to threefold during the late blastula stage (Shiokawa *et al.* 1986*a*). Therefore, it was suggested that ammonia might be a negatively controlling factor in the onset of rRNA synthesis in *Xenopus* embryogenesis (Shiokawa *et al.* 1986*a*).

The major question here is whether or not the weak bases, like ammonia, are really functioning as regulators of the commencement of rDNA expression during *Xenopus* embryogenesis; a difficult question to answer.

In the present experiments, in order to obtain more insight about the regulation of ribosome formation and the eligibility of ammonia as a negative regulator of the ribosome synthesis during *Xenopus* early development, we study the mode of action of the weak bases on rRNA synthesis, using *Xenopus* neurula cells as testing cells. We first obtained accurate dose–response curves of the inhibitory action for both ammonium chloride (AM) and trimethylammonium perchloride (TMA) using the condition that the assays were done after the weak bases established their inhibitory activity (conditions of 'pretreatment') within the testing cells. The results obtained strongly suggested that the inhibition of rRNA synthesis by weak bases is realized at the step of the formation of 40S pre-rRNA and is not due to the induction of the breakdown (wastage) of the 40S pre-rRNA and its subsequent processing intermediates. Also, it was strongly suggested that the inhibitory effect is not due to an interference with the energy supply, but probably due to a slight elevation of intracellular pH in the treated cells.

Materials and methods

Embryos and labelling of embryonic cells

Embryos of *Xenopus laevis* were obtained by injection of a gonadotropic hormone. Embryos were dejellied, dissociated into cells at yolk-plug and neurula stages (stages 12·5 and 20–21, respectively, Nieuwkoop & Faber, 1956), and labelled with 20, 100 or 200 μCi of [^{3}H]uridine (25 Ci mmole^{-1}) for varying lengths of time in 1·0 ml of the medium in the presence or absence of AM or TMA in complete Stearns' solution (Shiokawa & Yamana, 1967). In some of the experiments, actinomycin D was added at 10 μg ml^{-1} to follow the processing of rRNA-related components. In the experiment to see the effect of Na^+-free medium on rRNA synthesis, all the sodium salts in Stearns' complete medium were removed and these were replaced with 0·083 mole of choline chloride. The Na^+-free medium was buffered at pH 7·2 with 5 mM-Hepes–NaOH. Cultivation of neurula cells in the choline chloride medium caused a slight reduction in the [^{3}H]uridine incorporation, but did not induce any appreciable alteration in the pattern of RNA labelling in the control culture.

Extraction and fractionation of RNAs

Frozen cells were homogenized in 0·1 M-sodium acetate (pH 5·0) containing 10 μm ml^{-1} of bentonite and 0·5 % SDS (sodium dodecyl sulphate). Homogenates were mixed with phenol and then vigorously shaken at 20–25 °C for 1–2 h in a gyratory shaker. This 'partial-hot-phenol' treatment facilitated efficient deproteinization, yet did not induce degradation of RNA. RNA was precipitated from the aqueous phase with 0·2 M-NaCl and 2·5 vol of ethanol. Efficiency of the extraction of the labelled RNA was over 80 %.

RNAs equivalent to four embryos (approx. 16 μg) were electrophoresed on 10 cm gels of 0·5 % agarose–2·4 % polyacrylamide at 3 mA for either 1 or 2 h (Shiokawa *et al.* 1979). When electrophoresis was carried out for 2 h, 4S RNA passed out of gels, but rRNA-related components were retained within the gel and their separation was better than in 1 h electrophoresis. Gels were stained with methylene blue and exact locations of 18S and 28S rRNAs were marked. Gels were then sliced, RNAs were hydrolysed in 3 N-ammonia solution and radioactivity counted in ACS II scintillation mixture in a scintillation spectrometer (Shiokawa *et al.* 1979).

On the gel electrophoretic profiles obtained, the region where 40S pre-rRNA was migrated was determined by relating the reported relative molecular mass of the 40S pre-rRNA ($2{\cdot}6\times10^6$) to its expected distance of migration, using 18S and 28S mature rRNAs as references (0·7 and $1{\cdot}5\times10^6$, respectively) (Loening, Jones & Birnstiel, 1969; Slack & Loening, 1974; Misumi, Nishio, Shiokawa & Yamana, 1978). The 40S component detected has been shown to be processed into the mature rRNA (Misumi *et al.* 1978) and to be accumulated when cells were treated by an rRNA-processing inhibitor, tubercidin (Hagenbüchle, Schibler & Wyler, 1975; Shiokawa, 1984). The 30S pre-rRNA was identified according to its distance of migration relative to other rRNAs (Gelfand & Smith, 1983) and the previous data obtained by Schibler *et al.* (1976).

Preparation and fractionation of acid-soluble nucleotides

Acid-soluble materials were obtained by homogenizing dissociated cells, obtained from 300 neurulae, in 0·5 ml of 0·5 N-perchloric acid at 0 °C (Shiokawa *et al.* 1985). After being neutralized with KOH, aliquots of the soluble materials were subjected to high performance liquid chromatography equipped with ID column packed with Hitachigel No. 3013N (anion exchange resin; particle size 5–6 μm). Position of each of the components was determined using standard compounds.

Results

Reexamination of the dose–response effect of AM and TMA on the synthesis of 18S and 28S rRNAs under the 'pretreatment' conditions

Previously, we observed that 2·5 mM-ammonium dihydrogenphosphate inhibits rRNA synthesis in *Xenopus* neurula cells by 1 h after its administration (Shiokawa *et al.* 1986*a*). However, the exact timing

when the inhibition becomes maximum was not determined. In our previous experiments (Shiokawa *et al.* 1985; 1986*a*,*b*), we usually added [^{3}H]uridine and weak bases into the culture of neurula cells simultaneously. Therefore there was always a small, but distinct, amount of label incorporation in 18S and 28S rRNAs in neurula cells treated even at a relatively high dose of weak bases (5 mM). Probably, a certain amount of ^{3}H-UTP had been incorporated into rRNAs before weak bases became effective in inhibiting rRNA synthesis.

In the present experiments, we added [^{3}H]uridine to neurula cells 2·5 h after addition of AM or TMA and we labelled cells for the following 3 h in the continued presence of AM or TMA (conditions of 'pretreatment'). The 2·5 h of pretreatment was chosen because it was found that the incorporation of ammonia reached a plateau level at about 2·5 h after addition of ammonium salt (Shiokawa *et al.* 1986*a*).

In the RNA of cells labelled for 3 h, there were peaks of 28S rRNA, 18S rRNA (dotted peaks) and 4S RNA (Fig. 1A). There was also a fraction of heterogeneous high-molecular-weight RNA underneath the peaks of rRNAs (shaded). This profile was similar to those labelled for 5 h in our previous experiments (Shiokawa *et al.* 1986*b*). When cells were treated with 1 mM-AM under the pretreatment conditions, the inhibition in the labelling of 18S and 28S rRNA was relatively small (Fig. 1B). However, when cells were treated with 5 mM-AM, the inhibition was complete (Fig. 1C), yet the inhibition in the labelling of heterogeneous RNA and 4S RNA was relatively small.

Assays as in Fig. 1 were repeated and dose–response curves were obtained for AM and TMA as in Fig. 2. In the case of AM (Fig. 2A), rRNA synthesis was inhibited by 50% at about 2·2 mM, whereas 50% inhibition of 4S RNA synthesis was expected to be realized only at about 7 mM from the extrapolation of the curves obtained. The inhibition by AM was very small at doses less than 1 mM, yet it was complete at 3·5 mM. Thus, under the pretreatment conditions, the inhibition of rRNA synthesis became complete at doses lower than 5 mM. Therefore, we assume that the inhibitory activity on rRNA synthesis by weak bases can be demonstrated more clearly here than in our previous experiments in which [^{3}H]-uridine and weak bases were added simultaneously (Shiokawa *et al.* 1968*a*,*b*).

In our previous experiment (Shiokawa *et al.* 1986*a*), the change in the level of intraembryonic ammonia observed at MBT was relatively small (only two- to threefold). Therefore, the sharp transition in the inhibitory effect as shown in Fig. 2A provides evidence that a weak base, like ammonia, might be

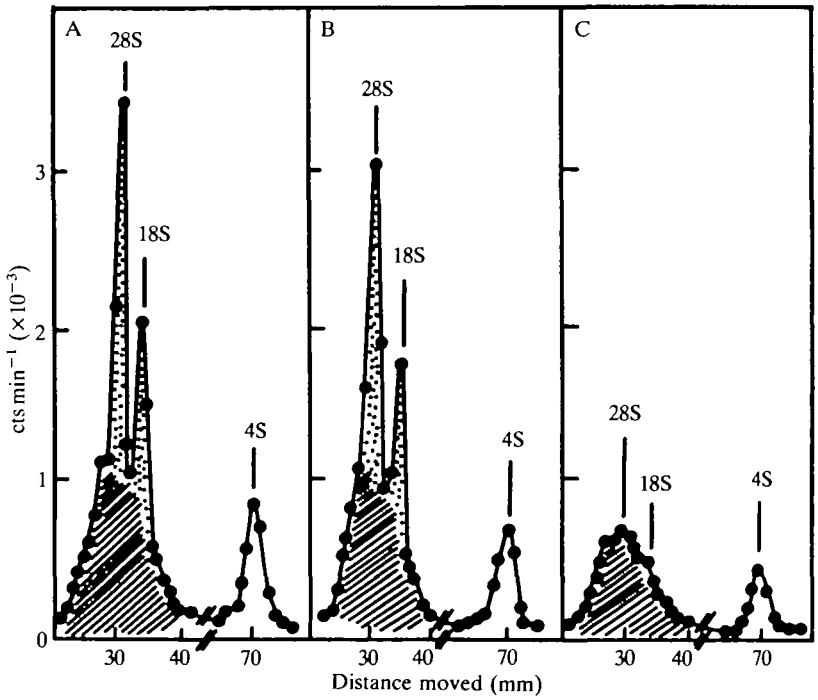

Fig. 1. Pattern of RNA labelled for 3 h under the 'pretreatment' conditions. (A) Control. Cells from 15 neurulae were treated with either (B) 1 mM- or (C) 5 mM-AM for 2·5 h, then labelled with 20 μCi of [^{3}H]uridine for 3 h in 1 ml of the medium. RNA was extracted and fractionated for 1 h on 0·5% agarose–2·4% polyacrylamide gels. Portions of gels that contained rRNA and 4S RNA were shown. Dotted area represents 28S and 18S rRNAs, and shaded area the heterogeneous nonribosomal high-molecular-weight RNA.

involved directly in the negative control of rRNA synthesis in *Xenopus* embryogenesis.

In the case of TMA, also, the dose–response curve obtained was quite sharp (Fig. 2B). From the comparison of the two dose–response curves it appears that the inhibiting effect of TMA on rRNA synthesis is significantly stronger than that of AM. While 50 % inhibition of rRNA synthesis was realized with TMA at about 0·6 mM, 2·2 mM was needed with AM as mentioned above.

When the kinetics of the dose–response effects of AM and TMA were compared on the same semilogarithmic scale, it became clearer that they are very similar to each other (Fig. 2C). This provides evidence that mode of action of these two weak bases is the same or at least very similar. The stronger action of TMA than AM may be related to the higher basicity of the former than the latter.

Uniform inhibition of 40S pre-rRNA, 30S rRNA intermediate, 18S and 28S mature rRNAs under the conditions of partial inhibition by AM and TMA

Recently, we found that 5 mM-AM or monomethylamine hydroperchloride completely inhibits the labelling of 18S and 28S mature rRNAs and 40S pre-rRNA in 2·5 h labelling. We obtained similar results when we tested the effect of pretreatment by 3 mM-AM on the pattern of 45 min labelled RNA (Shiokawa *et al.* 1986*a*). Since 40S pre-rRNA had been assumed to be the rDNA primary transcript (Wellauer & Dawid, 1974; Schibler *et al.* 1976), we interpreted the results as suggesting transcription-level inhibition of rDNA expression.

However, a possibility still remains that under the inhibited conditions 40S pre-rRNA might have been normally produced, but was degraded very quickly (wastage), or processed through aberrant pathways to be degraded very quickly.

Next, we selected the conditions of partial inhibition of rRNA synthesis, based on the dose–response curves (Fig. 2), and reexamined the effect of AM and TMA on the labelling of 40S pre-rRNA and other rRNA intermediates. When we labelled the control neurula cells for only 1 h (Fig. 3A), we obtained 40S pre-rRNA (black peak), 30S rRNA intermediate (shaded peak) and 18S rRNA (dotted peak), in addition to a large amount of heterogeneous RNA (not marked). We noticed the labelling of 30S rRNA intermediate in the present experiment, although in our previous experiment (Shiokawa *et al.* 1986*a*) we overlooked the 30S rRNA intermediate, since it migrated very close to 28S rRNA.

When we treated neurula cells with 2·6 mM-AM for 2·5 h and labelled the cells for 1 h (Fig. 3B), the inhibition was observed clearly in the labelling of all the rRNA-related components. The amounts of

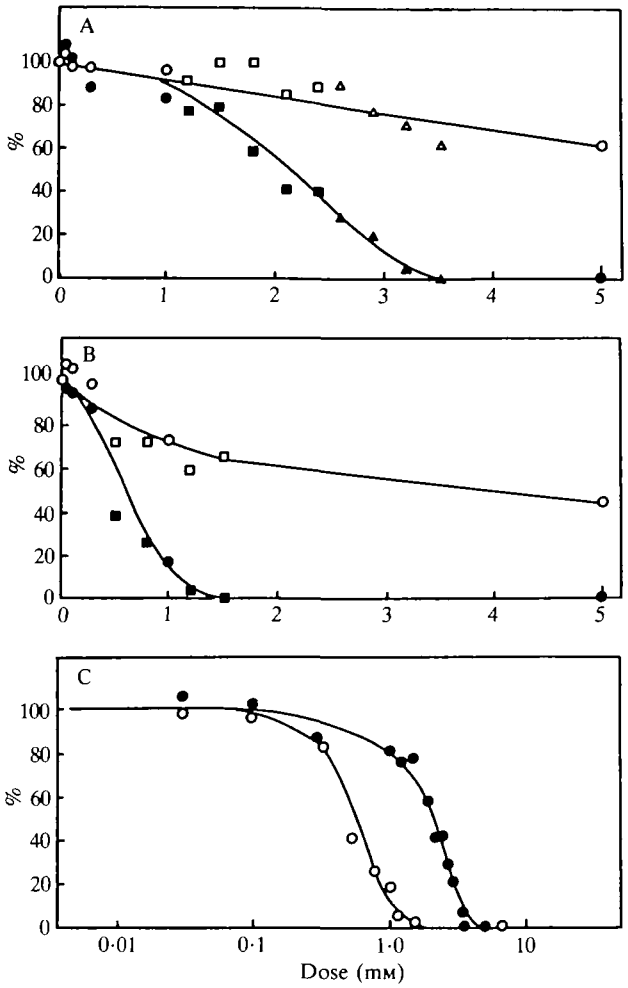

Fig. 2. Dose–response curves for inhibitory activity of AM and TMA on the synthesis of rRNA and 4S RNA in neurula cells. Effects of AM or TMA on rRNA synthesis were tested at various doses by labelling neurula cells with 20 μCi [^{3}H]uridine for 3 h under the pretreatment conditions as in Fig. 1. Amounts of [^{3}H]uridine incorporated into 28S plus 18S rRNAs and 4S RNA were determined. Values obtained were plotted taking the control values as 100 %. 4S RNA (open symbols), 28S plus 18S rRNAs (closed symbols). Different symbols are for different batches of embryos. (A) AM and (B) TMA. (C) Inhibition kinetics of the labelling of 18S plus 28S rRNAs. The relative inhibition was plotted on semilogarithmic scale on the abscissa. AM (●), TMA (○).

radioactivity incorporated were calculated based on the area under the respective RNA peak. The extents of the inhibition were 60 %, 59 % and 70 % for 40S pre-rRNA, 30S rRNA intermediate and 18S rRNA, respectively.

In this series of experiments, the neurula cells that had been pretreated with 2·6 mM-AM for 2·5 h were also labelled for 3 h (Fig. 3C,D). As in the experiment in Fig. 1, 18S and 28S mature rRNAs, but not 40S and 30S pre-rRNAs, were labelled in this 3 h labelling.

The inhibition of the labelling was calculated as 72 %, 69 % and 7 % for 28S and 18S rRNAs and 4S RNA, respectively (Fig. 3D). Therefore, it can be seen that the extents of the inhibition of 1 h-labelled 40S and 30S pre-rRNAs and 3 h-labelled 18S and 28S rRNAs were all quite similar.

In parallel to the above experiments, 1 mM-TMA was tested (2·5 h pretreatment followed by the labelling for 1 and 3 h) (profiles omitted). The percentage of the inhibition of labelling observed here was 67 %, 70 %, 75 %, 73 % and 8 % for 40S pre-rRNA, 30S rRNA intermediate, 28S rRNA, 18S rRNA and 4S RNA, respectively.

From these results, we may conclude that the extents of the inhibition observed at 40S pre-rRNA, 30S intermediate rRNA and 18S and 28S mature rRNAs were all the same. Therefore, it appears that the 40S pre-rRNA synthesized in the inhibited cells is processed normally and is not degraded during the processing.

Processing of 40S pre-rRNA to 18S and 28S mature rRNAs needs 30–60 min

To obtain direct evidence that weak bases do not interfere with rRNA processing, we next intended to follow the processing of the previously labelled 40S pre-rRNA. To do this, we first examined how 40S pre-rRNA is processed, using neurula cells and, in addition, yolk-plug cells.

When neurula cells were pulse labelled for 35 min, only 40S pre-rRNA and heterogeneous RNA were labelled (profile not shown, but see Fig. 5A, which was labelled according to the same protocol). When the labelling was stopped by adding actinomycin D at 10 $\mu g\,ml^{-1}$ and cells were incubated for varying lengths of time in the presence of the added actinomycin D, it was found that the 40S component disappeared in about 30 min and approximately the same amount of the label appeared in the fraction of 18S plus 28S mature rRNAs at 50 min after the

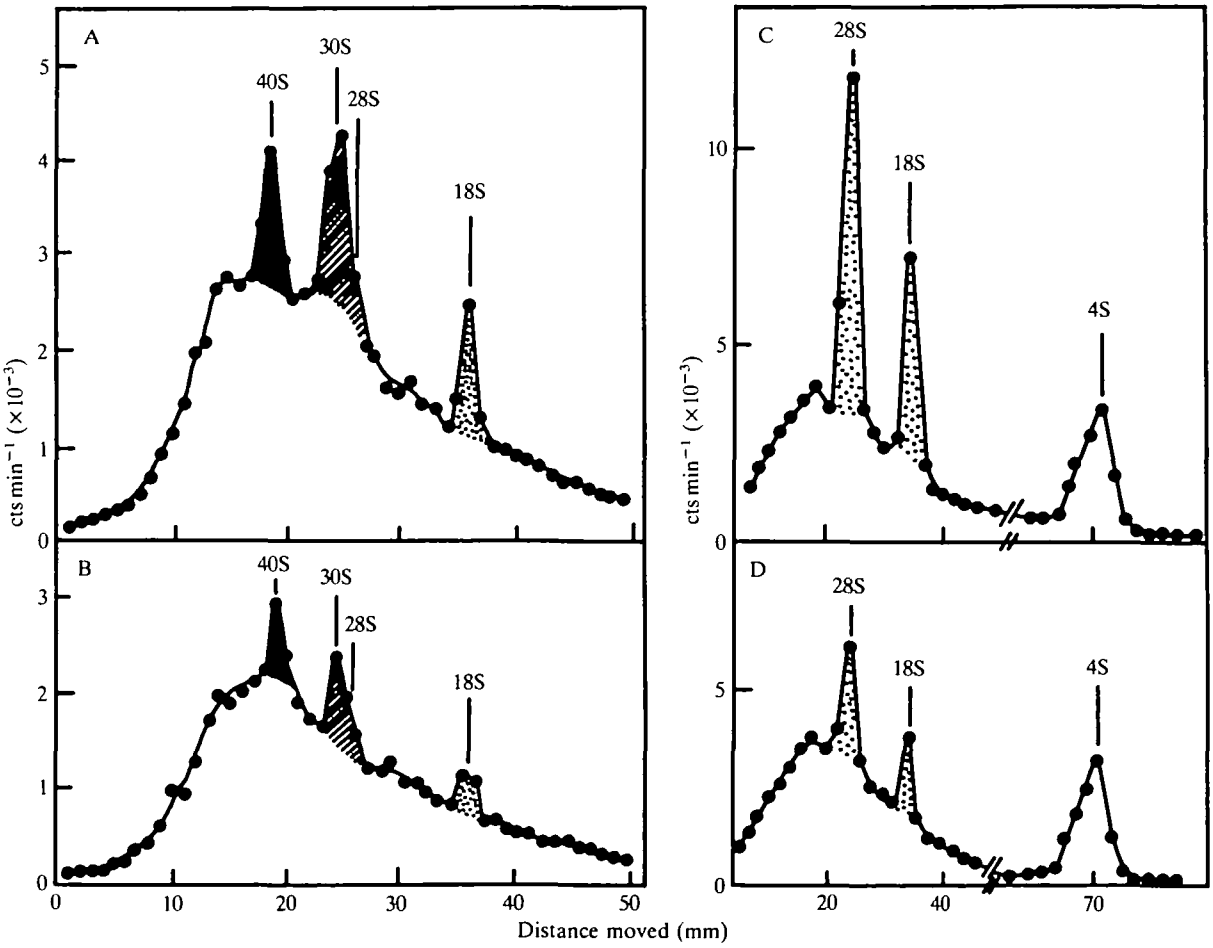

Fig. 3. Effects of AM on the synthesis of 40S and 30S pre-rRNAs and 18S and 28S rRNAs. Neurula cells from 15 embryos were treated with 2·6 mM-AM and then labelled for 1 h with 200 μCi [^{3}H]uridine (A,B), or for 3 h with 20 μCi [^{3}H]uridine (C,D). RNAs were extracted and electrophoresed for 2 h (A,B) or 1 h (C,D) as in Fig. 1. In (A) and (B), 4S was eluted out from the gel and is not shown. In (C) and (D), regions that contained rRNA and 4S RNA are shown. Black peak (40S RNA), shaded peak (30S RNA) and dotted peaks (28S and 18S RNAs). (A) and (C) Control; (B) and (D) inhibited by AM.

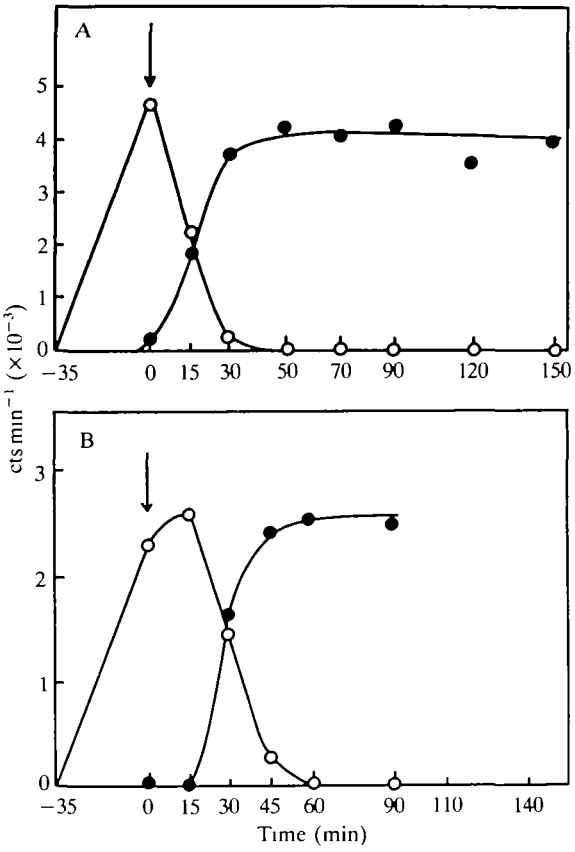

Fig. 4. Kinetics of the processing of 40S pre-rRNA into 18S and 28S rRNAs. Cells from 15 embryos at neurula stage (A) or yolk-plug stage (B) were pulse labelled with 100 μCi [^{3}H]uridine in 1 ml of the medium and then administered with actinomycin D at 10 μg ml^{-1}. Cells were then chased for varying lengths of time. RNAs were extracted and fractionated as in Figs 1, 3. Amounts of the label in 40S pre-rRNA and 18S plus 28S rRNAs were determined on the basis of the radioactivity peaks obtained, and plotted as a function of the time. Arrows indicate the time when actinomycin D was added.

actinomycin D addition (Fig. 4A). The processed 18S and 28S rRNAs were preserved without being degraded until 150 min in spite of the continued presence of actinomycin D (Fig. 4A).

Also, in the labelling of yolk-plug cells for 35 min, only 40S pre-rRNA and heterogeneous RNA were obtained. In this labelling, however, the radioactivity in the 40S pre-rRNA slightly increased during the chase for 15 min. 40S pre-rRNA disappeared completely by 60 min of chase and the corresponding amount of the label was recovered in the fraction of 18S plus 28S rRNA (Fig. 4B).

From these results, although the time course differs slightly depending on the stages of the embryonic cells used, 30–60 min is needed for the pulse-labelled 40S pre-rRNA to be processed completely into 18S and 28S rRNAs.

Noneffectiveness of AM and TMA on the processing of previously labelled 40S pre-rRNA

Based on the above results we pulse labelled neurula cells for 35 min and tested the effect of AM or TMA on the processing of the prelabelled 40S pre-rRNA using 10 μg ml^{-1} of actinomycin D as a transcription inhibitor. The chase period was determined to be 2 h, since the above experiments showed that 30–60 min was necessary for the complete processing of the pulse-labelled 40S pre-rRNA.

When neurula cells were pulse labelled for 35 min, the RNA of the zero-time culture again contained only 40S pre-rRNA and heterogeneous mRNA-like RNA as pointed out above (Fig. 5A). When cultures, pulse labelled for 35 min, were chased for 2 h in the presence of actinomycin D, the label in the 40S pre-rRNA completely disappeared and was recovered in 18S and 28S mature rRNAs (Fig. 5B). Such recovery of the label in 18S and 28S rRNAs occurred also in the presence of 10 mM-AM (Fig. 5C) or TMA (Fig. 5D). The total amount of the label in rRNA as well as that in heterogeneous RNA did not greatly increase during the chase, implying that actinomycin D almost completely suppressed label incorporation very shortly after its addition.

In these experiments, cultures were also prepared which were pulse labelled for 35 min and then immediately administered with either AM (Fig. 5F) or TMA (Fig. 5G), but not actinomycin D. Since actinomycin D was not added, the incorporation continued to occur during the following 2 h of incubation. This is clearly shown by a large increase in the amounts of heterogeneous RNA in all the cultures (Fig. 5E–G). However, very interestingly, there was practically no label accumulation in rRNA in the cultures treated by AM (Fig. 5F) or TMA (Fig. 5G). It is noted here that there must be some small amount of accumulation of the label in rRNA which was derived from the pulse-labelled 40S pre-rRNA. However, the large amount of the label that accumulated in heterogeneous RNA during the continued labelling obscured the peaks of the processed rRNAs. Thus, it is clearly shown that both AM and TMA inhibited rRNA synthesis almost completely.

Based on these results, we may now conclude that AM and TMA inhibit rRNA synthesis almost completely but do not inhibit the processing of 40S pre-rRNA. Also, the finding of almost complete quantitative recovery of the label in 40S pre-rRNA into two mature rRNAs in the presence of AM or TMA excludes the possibility of aberrant processing leading to the degradation of processed rRNAs.

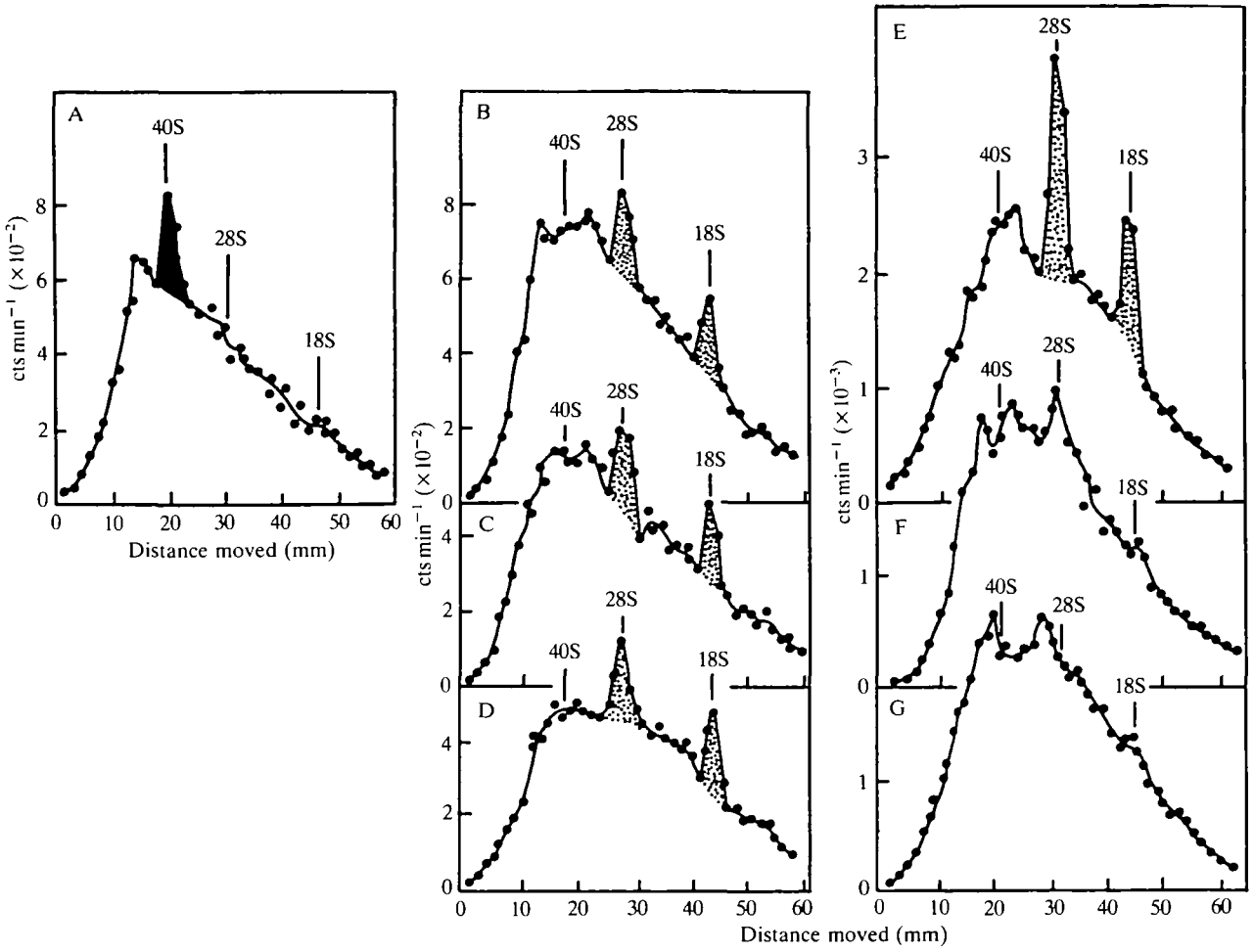

Fig. 5. Effects of AM and TMA on the processing of 40S pre-rRNA labelled before addition of the weak bases. Cultures of cells from 15 neurulae were pulse labelled with 200 μCi [^{3}H]uridine for 35 min. One culture was then immediately frozen as a zero-time sample (A). Three cultures (B,C,D) were administered with 10 μg ml^{-1} actinomycin D and then with either 10 mM-AM (C), 10 mM-TMA (D) or nothing (B). Other three cultures (E,F,G) were administered only with either 10 mM-AM (F), 10 mM-TMA (G) or nothing (E). Six cultures (B to G) were all incubated for another 2 h. RNAs were extracted and electrophoresed as in Fig. 3A. Black peak (40S RNA) and dotted peaks (28S and 18S RNAs).

Disappearance of the inhibitory effect of AM and TMA in the Na^+-free medium

We tested the effect of 3 mM-AM and 1·5 mM-TMA in neurula cells in normal Stearns' medium and reconfirmed that these were effective in strongly inhibiting rRNA synthesis (over 75 % for both AM and TMA) under the pretreatment conditions (Fig. 6A–C). Simultaneously, using neurula cells of the same batch, we tested the effects of the same concentration of these weak bases in a medium in which all the Na^+ was replaced by choline ions.

Results of the labelling experiment showed that the inhibitory effects of both AM (Fig. 6E) and TMA (Fig. 6F) were completely abolished in the medium whose Na^+ had been replaced by choline ions. Therefore, the present results strongly suggest that for the inhibitory effect of both AM and TMA to appear, Na^+ is needed in the surrounding medium.

Effect of AM and TMA on ATP level

We measured the level of ATP and other ribonucleotides in the acid-soluble fraction of neurula cells after treating them for 3 h with 3 mM-AM (Fig. 7B) or 1·5 mM-TMA (Fig. 7C). The results obtained show that the level of ATP as well as other ribonucleotide triphosphates remained unchanged. In the control culture the amount of the ATP detected in one-embryo-equivalent neurula cells was about 1000 pmole, which is consistent with our previous determination (Tashiro, Misumi, Shiokawa & Yamana, 1983). Therefore, neither AM nor TMA appear to disturb the energy-generating system in *Xenopus* neurula cells.

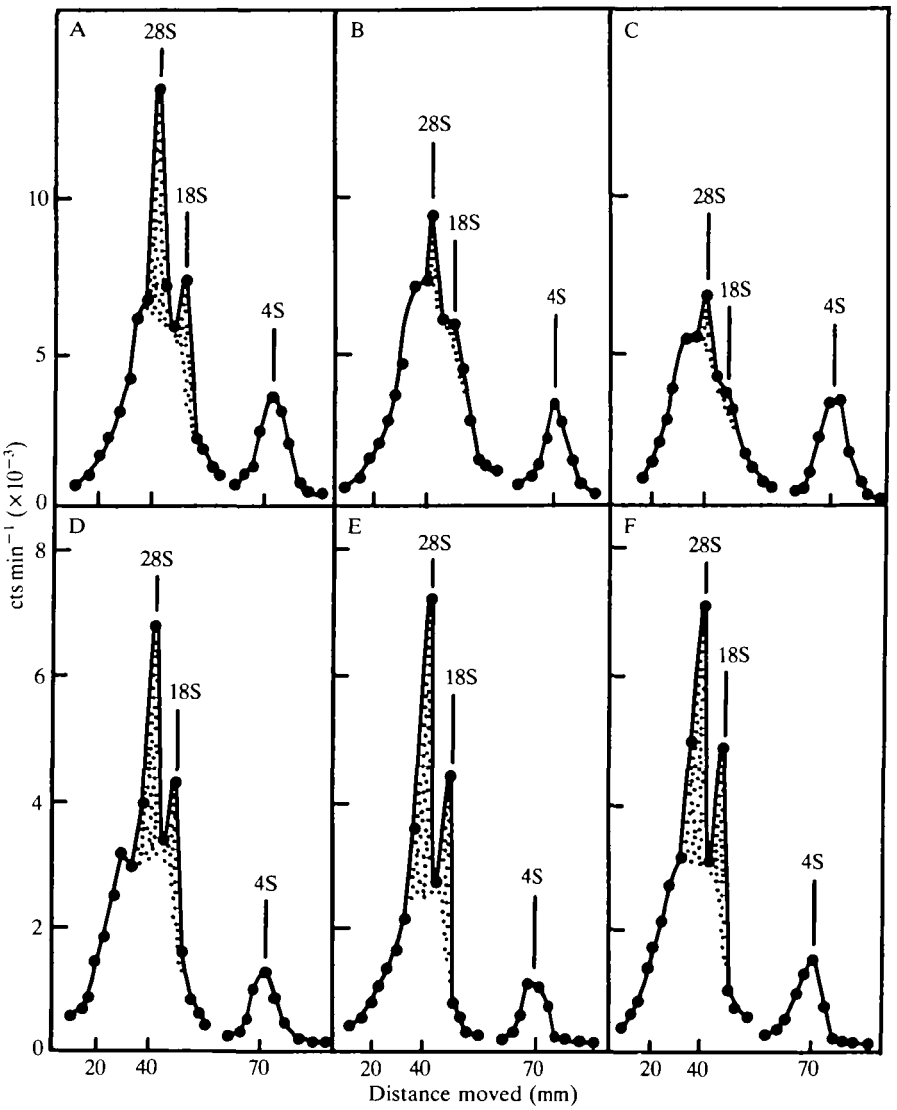

Fig. 6. Disappearance of the inhibitory effect of AM and TMA in the medium in which Na^+ was replaced by choline ion. Cells from 15 neurulae were treated for 2·5 h either in the normal medium (A,B,C) or in Na^+-free medium (D,E,F) with 3 mM-AM (B,E) or 1·5 mM-TMA (C,F). (A,D) Controls. Cells were then labelled with 20 μCi [^{3}H]uridine for 3 h in the continued presence of the weak bases. RNAs were extracted and electrophoresed as in Fig. 1. Dotted peaks are 28S and 18S RNAs.

Discussion

Present results showed that the inhibition of rRNA synthesis by AM and TMA is due to the inhibition of the formation of 40S pre-rRNA, and not to the induction of its breakdown (wastage), or to the induction of its aberrant processing which would lead to degradation of the processed rRNAs. These conclusions were drawn mainly from two lines of evidence. The first is the uniform inhibition of the labelling of 40S pre-rRNA, 30S rRNA intermediate and 18S and 28S mature rRNAs under the conditions of the partial inhibition. The second is the non-inhibited processing of prelabelled 40S pre-rRNA into 18S and 28S mature rRNAs in the presence of a very high dose (10 mM) of AM and TMA.

However, we need to evaluate the validity of the second line of evidence. In this chase experiment, designed to study the effect of weak bases on the processing of 40S pre-rRNA, we tested the pattern of RNA labelling 2 h after the administration of actinomycin D. If the processing had been finished before the weak bases became effective, we could not have tested the effect of the weak bases on the processing. As shown in the experiment in Fig. 4A,B, however, 30–60 min is needed for the complete processing of 40S pre-rRNA in *Xenopus* embryonic cells. On the other hand, AM and TMA at 10 mM inhibited rRNA synthesis immediately after the administration. We

know that the label incorporation into RNA (especially into rRNA) in neurula cells proceeds linearly with time (Shiokawa & Yamana, 1967; Shiokawa *et al.* 1986*a*). Therefore, if AM or TMA were not effective for the first 30–60 min, there should be a significant accumulation of the label (probably one-fourth to one-half of the control level) in 18S and 28S rRNAs in the AM- or TMA-treated cells. However, this was not the case. Therefore, the processing of the prelabelled 40S pre-rRNA appeared to have occurred while AM or TMA was effective in the treated cells. Thus, the conclusion that AM or TMA does not interfere with the processing of 40S pre-rRNA appears to be valid.

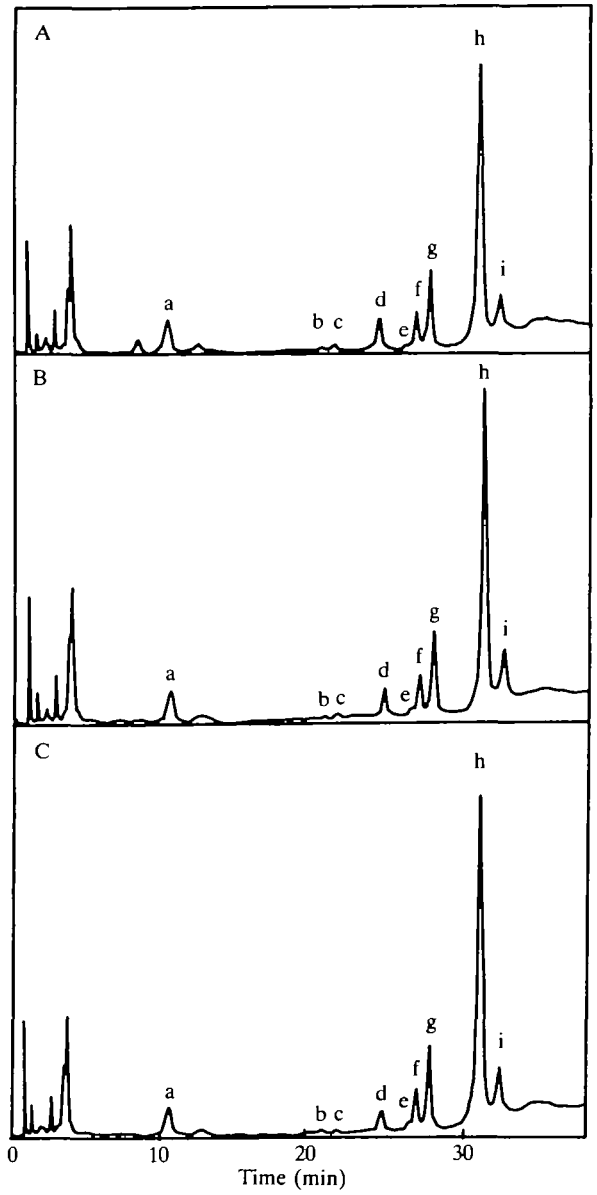

Fig. 7. Effects of AM and TMA on the level of ATP and other ribonucleotides. Cells from 300 neurulae were treated with 3 mM-AM or 1·5 mM-TMA for 3 h. Cells were homogenized in 0·5 N-perchloric acid, and acid-soluble fraction was extracted. Aliquots were analysed by high performance liquid chromatography and profiles of u.v. absorption at 260 nm were obtained. Peaks are a (AMP), b (CDP), c (UDP), d (ADP), e (GDP), f (CTP), g (UTP), h (ATP) and i (GTP). (A) Control, (B) treated with AM and (C) treated with TMA.

The expression of rDNA has long been assumed to begin with the formation of 40S pre-rRNA (Wellauer & Dawid, 1974; Schibler *et al.* 1976). However, very recently it has been reported that probably entire spacer and coding regions (except a small stretch of 212 bp) are transcribed (de Winter & Moss, 1986). At present, rRNA appears to be transcribed as a giant molecule and appears to be converted quickly to a relatively 'stable' 40S pre-rRNA, which is then processed into 18S and 28S rRNAs *via* 30S intermediate (Wellauer & Dawid, 1974; Schibler *et al.* 1976). Since the present results revealed that weak bases inhibit the formation of 40S pre-rRNA, the real mechanism of their action may be the inhibition of rDNA transcription or post-transcriptional processing very shortly after the transcription but before the step of the formation of 40S pre-rRNA.

As for the mode of action of weak bases, it has already been suggested that a slight elevation of intracellular pH is important (Webb & Charbonneau, 1986; Garcia-Sato, La Torre & Darszon, 1985; Guerrier, Brassart, David & Moreau, 1986; Williams, Elder & Sussman, 1986; Dube & Epel, 1986; Aerts, Durston & Moolenaar, 1985; Wasserman, Houle & Samuel, 1984; Houle & Wasserman, 1983). In some of these papers, it has been shown also that removal of Na^+ from the medium inhibits a slight elevation of pH, thereby abolishing the pH-mediated effect of weak bases (Wasserman & Houle, 1984; Stith &

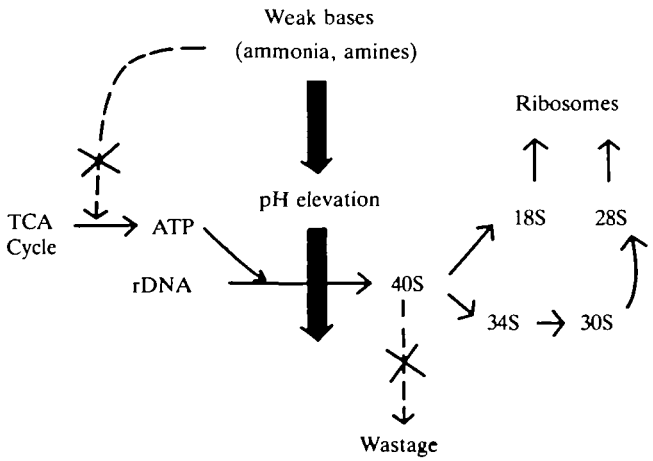

Fig. 8. A working hypothesis on the mode of action of weak bases on rRNA synthesis. Weak bases do not interfere with ATP generation (upper X) and do not induce degradation of 40S pre-rRNA (lower X), but block the formation of 40S pre-rRNA (lower large arrow) probably at the transcriptional level *via* a slight elevation of intracellular pH (upper large arrow).

Maller, 1985). In our present results, we showed very clearly that the inhibitory effect of weak bases on rRNA synthesis was completely abolished in Na^+-free medium. Therefore, it appears that the inhibition observed here may also be realized by a slight increase in the intracellular pH.

Finally, it is known that when the level of intracellular ammonia is increased, oxaloacetic acid is transaminated into aspartic acid and TCA cycle is inhibited. This causes a decrease in the level of ATP. However, present results showed that there was no decrease in the level of ATP in the weak base-treated cells. We may conclude that the effect of ammonium salt on rRNA synthesis is not *via* inhibition of the TCA cycle. This is consistent with the fact that various amines, which cannot bind to oxaloacetic acid, are also quite effective in inhibiting rRNA synthesis (Shiokawa *et al.* 1986*b*).

Based on the results summarized above, a working hypothesis here is that both ammonia and amines suppress rDNA transcription, or a step very close to the transcription, probably *via* a process that is blocked by a slight increase in the intracellular pH but not *via* interference in energy supply (Fig. 8).

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References

AERTS, R. J., DURSTON, A. J. & MOOLENAAR, W. H. (1985). Cytoplasmic pH and regulation of the *Dictyostelium* cell cycle. *Cell* **43**, 653–657.

BIRD, A., TAGGART, M. & MACLEOD, D. (1981). Loss of rDNA methylation accompanies the onset of ribosomal gene activity in early development of *X. laevis*. *Cell* **26**, 381–390.

BROWN, D. D. & GURDON, J. B. (1964). Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc. natn. Acad. Sci. U.S.A.* **51**, 139–146.

COOPER, H. L. (1968). Ribonucleic acid metabolism in lymphocytes stimulated by phytohemagglutinin II. Rapidly synthesized ribonucleic acid and the production of ribosomal ribonucleic acid. *J. biol. Chem.* **243**, 34–43.

CRAMPTON, J. M. & WOODLAND, H. R. (1979). Isolation from *Xenopus* embryonic cells of a factor which stimulates ribosomal RNA synthesis by isolated nuclei. *Devl Biol.* **70**, 467–478.

DE WINTER, R. F. J. & MOSS, T. (1986). The ribosomal spacer in *Xenopus laevis* is transcribed as part of the primary ribosomal RNA. *Nucleic Acids Res.* **14**, 6041–6051.

DUBE, F. & EPEL, D. (1986). The relation between intracellular pH and rate of protein synthesis in sea urchin eggs and the existence of a pH-independent event triggered by ammonia. *Expl Cell Res.* **162**, 191–204.

GARCIA-SOTO, J., LA TORRE, L. D. & DARSZON, A. (1985). Increasing intracellular pH of sea urchin sperm with NH_4Cl induces Ca^{2+} uptake and acrosome reaction in the absence of egg jelly. *FEBS Letters* **190**, 33–36.

GELFAND, R. A. & SMITH, L. D. (1983). RNA stabilization and continued RNA processing following nuclear dissolution in mature *Xenopus laevis* oocytes. *Devl Biol.* **99**, 427–436.

GUERRIER, P., BRASSART, M., DAVID, C. & MOREAU, M. (1986). Sequential control of meiosis reinitiation by pH and Ca^{2+} in oocytes of the prosobranch Mollusk *Patella vulgata*. *Devl Biol.* **114**, 315–324.

GURDON, J. B. & BROWN, D. D. (1965). Cytoplasmic regulation of RNA synthesis and nucleolar formation in developing embryos of *Xenopus laevis*. *J. molec. Biol.* **12**, 27–35.

HAGENBÜCHLE, O., SCHIBLER, U. & WYLER, T. (1975). Intermolecular renaturation of hairpin loops leads to stable and specific aggregates of 28-S rRNA and precursor rRNAs from *Xenopus laevis*. *Eur. J. Biochem.* **60**, 73–82.

HOULE, J. G. & WASSERMAN, W. J. (1983). Intracellular pH plays a role in regulating protein synthesis in *Xenopus* oocytes. *Devl Biol.* **97**, 302–312.

LIEBERMAN, I., ABRAMS, R. & OVE, P. (1963). Changes in the metabolism of ribonucleic acid preceding the synthesis of deoxyribonucleic acid in mammalian cells cultured from the animal. *J. biol. Chem.* **238**, 2141–2149.

LOENING, U. E., JONES, K. W. & BIRNSTIEL, M. L. (1969). Properties of the ribosomal RNA precursor in *Xenopus laevis*; Comparison to the precursor in mammals and in plants. *J. molec. Biol.* **45**, 353–366.

MISUMI, Y., NISHIO, Y., SHIOKAWA, K. & YAMANA, K. (1978). RNA metabolism in primary cultures of *Xenopus laevis* kidney cells. III. Processing of rRNA precursor in growing and resting cells. *Expl Cell Res.* **114**, 239–245.

NEWPORT, J. & KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675–686.

NIEUWKOOP, P. D. & FABER, J. (1956). *Normal Table of* Xenopus laevis (*Daudin*). Amsterdam: North-Holland Publ. Co.

REEVES, R. (1978). Structure of *Xenopus* ribosomal gene chromatin during changes in genomic transcription rates. *Cold Spring Harbor Symp. Quant. Biol.* **42**, 709–722.

RUSSELL, D. H. (1983). Microinjection of purified ornithine decarboxylase into *Xenopus* oocytes selectively stimulates ribosomal RNA synthesis. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1318–1321.

SCHIBLER, U., HAGENBÜCHLE, O., WYLER, T., WEBER, R., BOSELEY, P., TELFORD, J. & BIRNSTIEL, M. L. (1976). The arrangement of 18-S and 28-S ribosomal ribonucleic acids within the 40-S precursor molecule of *Xenopus laevis*. *Eur. J. Biochem.* **68**, 471–480.

SHIOKAWA, K. (1984). Inhibitor of ribosomal RNA synthesis in *Xenopus laevis* embryos. VII. Inhibition of 40S pre-rRNA synthesis in *Xenopus* neurula cells. *Cell Struct. Func.* **9**, 97–102.

SHIOKAWA, K., KAWAZOE, Y., NOMURA, H., MIURA, T., NAKAKURA, N., HORIUCHI, T. & YAMANA, K. (1986*a*). Ammonium ion as a possible regulator of the commencement of rRNA synthesis in *Xenopus laevis* embryogenesis. *Devl Biol.* **115**, 380–391.

SHIOKAWA, K., KAWAZOE, Y., TASHIRO, K. & YAMANA, K. (1986*b*). Effects of various ammonium salts, amines, polyamines and α-methylornithine on rRNA synthesis in neurula cells of *Xenopus laevis* and *Xenopus borealis*. *Cell Differ.* **18**, 101–108.

SHIOKAWA, K., KAWAZOE, Y. & YAMANA, K. (1985). Demonstration that inhibitor of rRNA synthesis in "charcoal-extracts" of *Xenopus* embryos is artifactually produced ammonium perchlorate. *Devl Biol.* **112**, 258–260.

SHIOKAWA, K., MISUMI, Y. & YAMANA, K. (1981*a*). Demonstration of rRNA synthesis in pre-gastrular embryos of *Xenopus laevis*. *Dev. Growth, Differ.* **23**, 579–587.

SHIOKAWA, K., MISUMI, Y., YASUDA, Y., NISHIO, Y., KURATA, S., SAMESHIMA, M. & YAMANA, K. (1979). Synthesis and transport of various RNA species in developing embryos of *Xenopus laevis*. *Devl Biol.* **68**, 503–514.

SHIOKAWA, K., TASHIRO, K., MISUMI, Y. & YAMANA, K. (1981*b*). Non-coordinated synthesis of RNA's in pregastrular embryos of *Xenopus laevis*. *Dev. Growth, Differ.* **23**, 589–597.

SHIOKAWA, K. & YAMANA, K. (1967). Pattern of RNA synthesis in isolated cells of *Xenopus laevis* embryos. *Devl Biol.* **16**, 368–388.

SLACK, J. M. W. & LOENING, U. E. (1974). 5-'Ends of ribosomal and ribosomal precursor RNAs from *Xenopus laevis*. *Eur. J. Biochem.* **43**, 59–67.

SPADAFORA, C. & RICCARD, P. (1985). Different conformations of ribosomal DNA in active and inactive chromatin in *Xenopus laevis*. *J. molec. Biol.* **186**, 743–758.

STITH, B. J. & MALLER, J. L. (1985). Increased intracellular pH is not necessary for ribosomal protein S6 phosphorylation, increased protein synthesis, or germinal vesicle breakdown in *Xenopus* oocytes. *Devl Biol.* **107**, 460–469.

TASHIRO, K., MISUMI, Y., SHIOKAWA, K. & YAMANA, K. (1983). Determination of the rate of rRNA synthesis in *Xenopus laevis* triploid embryos produced by low-temperature treatment. *J. exp. Zool.* **225**, 489–495.

WASSERMAN, W. J. & HOULE, J. G. (1984). The regulation of ribosomal protein S-6 phosphorylation in *Xenopus* oocytes: A potential role for intracellular pH. *Devl Biol.* **101**, 436–445.

WASSERMAN, W. J., HOULE, J. G. & SAMUEL, D. (1984). The maturation response of stage IV, V, and VI *Xenopus* oocytes to progesterone stimulation *in vitro*. *Devl Biol.* **105**, 315–324.

WEBB, D. J. & CHARBONNEAU, M. (1986). Weak bases inhibit cleavage and embryogenesis in the amphibians, *Xenopus* (toad) and *Pleurodeles* (newt) and echinoderms *Paracentrotus sphaerechinus* (sea urchin) and asterias (starfish). *Cell Differ.* **20**, 33–44.

WELLAUER, P. K. & DAWID, I. B. (1974). Secondary structure maps of ribosomal RNA and DNA: I. Processing of *Xenopus laevis* ribosomal RNA and structure of single-stranded ribosomal DNA. *J. molec. Biol.* **89**, 379–395.

WILLIAMS, G. B., ELDER, E. M. & SUSSMAN, M. (1986). NH_3 and propionate modulate the morphological response of aggregation-competent *Dictyostelium discoideum* to cAMP. *Differentiation* **31**, 92–99.

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