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Zoologia. — *On protein synthesis in* Xenopus laevis *embryos* (*). Nota di FIORENZA DE BERNARDI e ANNA MARIA BOLZERN, presentata (**) dal Socio S. RANZI.

RIASSUNTO. — Embrioni di *X. laevis* allo stadio di neurula precoce sono stati divisi in due parti, dorsale e ventrale, sono stati trattati con actinomicina D e incubati con leucina ^{14}C per un periodo di 18 ore, fino al raggiungimento dello stadio di risposta muscolare.

Nella regione dorsale si osserva una incorporazione maggiore nei trattati rispetto ai controlli a livello di parecchie frazioni polisomali. Conducendo tre serie di incorporazioni brevi in tre stadi successivi del lungo periodo di sviluppo prima considerato si osserva che le varie classi di polisomi appaiono essere attive in tempi successivi, prima le più leggere (st. 15), poi le più pesanti (st. 25).

Se si tagliano gli embrioni allo stadio di bottone codale (22 di N. e F.) in tre parti, e si fanno incubare i frammenti con leucina ^{14}C si osserva che le frazioni polisomali più pesanti sono attive nel capo, mentre le più leggere sono attive nella regione del dorso. L'effetto « superinduttivo » della actinomicina viene interpretato come conseguenza dell'attivazione di messaggeri stabili, presenti nella regione dorsale fin da stadi precoci di sviluppo.

La regione ventrale diviene attiva nella sintesi proteica solo a partire dallo stadio di bottone codale: in essa non si osserva una « superinduzione » così marcata come nella regione dorsale: si presume perciò che i messaggeri per il differenziamento istologico vengano formati durante la chiusura del tubo neurale e vengano tradotti entro breve tempo.

The synthesis of RNA and proteins during early amphibian development has already been studied; nevertheless the problem of when the mRNAs for histological differentiation are synthetized in the embryo is still open. Actually, while it has been verified that the synthesis of ribosomal RNA starts at the early gastrula stage (Brachet and Malpoix, 1971), on the contrary, other RNAs—particularly the messengers or their precursors—are already being synthetized during the segmentation stages, mostly in the endodermic cells (Woodland and Gurdon, 1968).

Denis (1968) thinks that mRNA with rapid turnover is produced during every developmental stage, and that other long-lived mRNAs, which were found at the neurula stage, direct the differentiation. According to Flickinger (1970) more stable RNAs were found in the dorsal region of frog embryo at neurula and tail bud stages. The final evidence that stable, long-lived mRNA are able to direct protein synthesis for a long time was obtained by Gurdon *et al.* (1974) by injecting globin mRNA from mammal into fertilized eggs of *Xenopus*.

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In a previous work (Leonardi Cigada *et al.*, 1975) we tackled the problem of when the synthesis of messengers which direct the histological differentiation starts by cutting the embryos into two parts at various developmental stages, by blocking the RNA synthesis with Actinomycin D and by labeling the embryos with precursors of RNA and protein synthesis. The dorsal region of the embryos cut and treated with actinomycin D from the early neurula stages on showed an increase in ^{14}C leucine incorporation in the total proteins (precipitable TCA.) Therefore we carried out this research in order to determine whether the increased incorporation is to be ascribed to the whole translational system or, as the results seem to show, there are some polysomal fractions active in translating previously formed mRNA even in the presence of Actinomycin D.

The methods of collection and breeding of the embryos have already been described (Leonardi Cigada *et al.*, 1968, 1975). 45 *Xenopus laevis* embryos at stage 15 of Nieuwkoop and Faber (early neural folds) were cut in to two regions: dorsal and ventral. The cut was made following the archenteron cavity so as to leave the presumptive zone of the head, somites, notochord and nervous system in the dorsal region. The two groups of fragments were placed in 5 ml of Holtfreter solution containing streptomycin (0.1 μg/ml) and penicillin (200 I.U./ml). The embryos were treated with Actinomycin D (1 μg/ml) and incubated with 2 μC/ml of ^{14}C leucine (Radiochemical Centre, Amersham, s.a. 348 mC/mmole) at 18 °C as indicated.

A second series of experiments was carried out on embryos at tail-bud stage (22 of Nieuwkoop and Faber) cut into three parts: head, dorsal region (neural tube, notochord and somites) and ventral region (endoderm). The fragments were then cultured as previously described.

We carried out four kinds of incubations: incubation of 18 hours and shorter incubations of 45 minutes in three different moments of this long period for both series of experiments.

At the end of the incubation period the fragments were washed in Holtfreter solution containing unlabeled leucine and the polysomes were prepared. The fragments were rinsed and homogenized in one milliliter of buffer containing 0.3 M KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, 4 μg/ml polyvinyl sulphate and 50 μl of 10% sodium deoxycholate in water solution. This high concentration of KCl dissociates the monosomes which are not associated with mRNA, and ensures an optimal recovery and a minimum degradation of the polysomes (Woodland, 1974).

The samples were centrifuged at 12,000 *g* for 10 minutes at 4 °C and the supernatant was layered onto sucrose linear gradients 15–40% in buffer. The gradients were centrifuged for 3 h at 4 °C in Spinco SW 25.1 at 25,000 r.p.m. The fractions (1 ml) were collected and radioactivity was determined by scintillation counting with toluene-Triton (2 : 1) cocktail.

Results. The sedimentation patterns obtained from the dorsal regions of the embryos cut at the early neurula stage (15 of Nieuwkoop and Faber) and incubated for 18 hours (Fig. 1 a,) i.e. up to the muscular response stage (25 of N. and F.) show four main classes of polysomes which correspond to the fractions 15, 18, 21 and 25. In almost all fractions the actinomycin-treated fragments incorporate more than the controls do. This result confirms the data which have previously been obtained (Leonardi Cigada *et al.*, 1975), by measuring the ^{14}C leucine incorporated in the total proteins.

Therefore we carried out three series of short incubations (45 minutes), respectively at the beginning (st. 15), in the middle (st. 22) and at the end

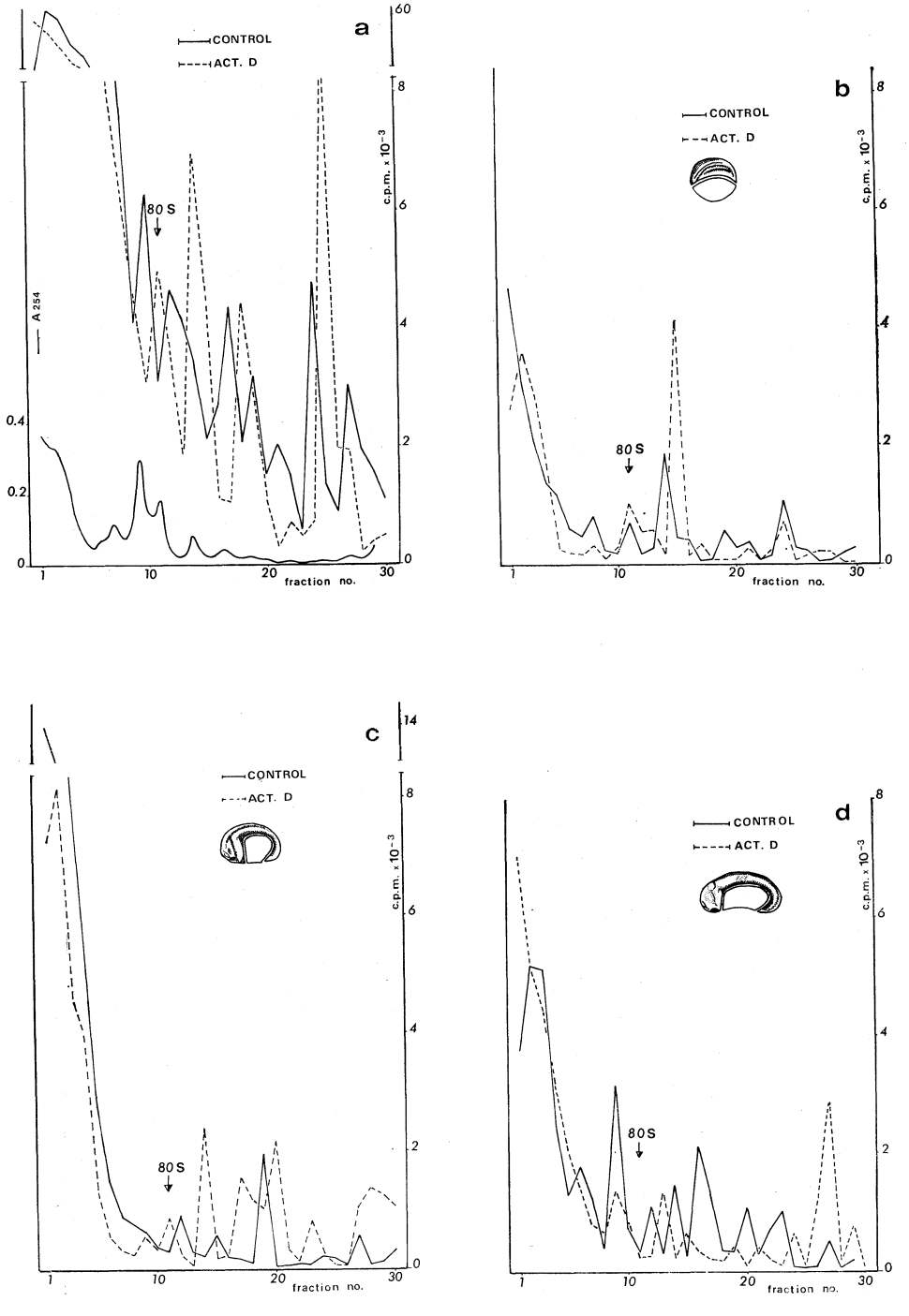

Fig. 1. – Sedimentation patterns of polysomes from the dorsal region of *Xenopus laevis* embryos cut at the early neurula stage (15 of N. and F.) and incubated with ^{14}C leucine for 18 hours (*a*) and 45 minutes (*b*, *c*, *d*) at neurula, tail-bud and muscular response stages.

(st. 25) of the incubation time (18 hours) in order to see when the various fractions were more active. The embryos were always cut into two parts at stage 15 and treated with actinomycin until the incubation stage was reached. An increase in the activity of fraction 15 (corresponding to light polysomes, about 120 S) is observed in the embryos cut at the early neurula stage and incubated for 45 minutes; this fraction appears to be highly superinduced in the treated embryos, but on the contrary other fractions are scarcely active (Fig. 1 b). Fraction 15 is still active at the tail-bud stage, while the incorpo-

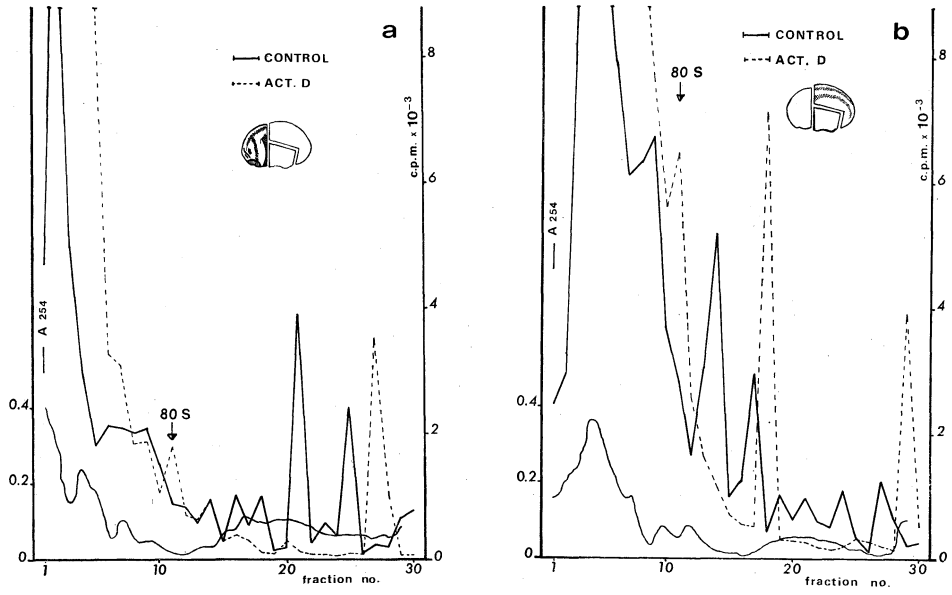

Fig. 2. – Sedimentation patterns of polysomes from the head region (*a*) and from the dorsal region (*b*) of *Xenopus laevis* embryos cut at tail-bud stage (22 of N. and F.) and incubated for 18 hours.

ration increases in fractions 18 and 21 (Fig. 1 c). At muscular response stage fraction 25 is particularly active and superinduced, the lightest fractions showing, on the contrary, a low activity (Fig. 1 d).

Further experiments were carried out on embryos at stage 22, when the head is differentiated; the embryos were cut into three regions: head, dorsal region and ventral region; the fragments were incubated for 18 hours. The same fractions, which had already been found in those embryos in which the mRNA synthesis was stopped at the neurula stage, were then observed. The heavy fractions are active in the head, while the lighest fractions are active in the dorsal region (Fig. 2). These fractions are likely to coincide with some stable mRNA synthesized at an early stage and perhaps active during the synthesis of some specific proteins for histological differentiation. On the contrary, fraction 28–29 appears to be highly superinduced both in the head and in the dorsal region.

The following experiment was carried out to confirm that the heaviest fractions are active in the head, while the lightest fractions are active in the dorsal region. At the early neurula stage the embryos were cut into two parts and were treated with actinomycin. When the embryos reached stage 22, the dorsal region was separated from the head, which is recognizable at this moment, and the two groups of fragments were incubated with labeled leucine for 45 minutes. We again found fraction 21 in the head and fraction 15 in the dorsal region (Fig. 3).

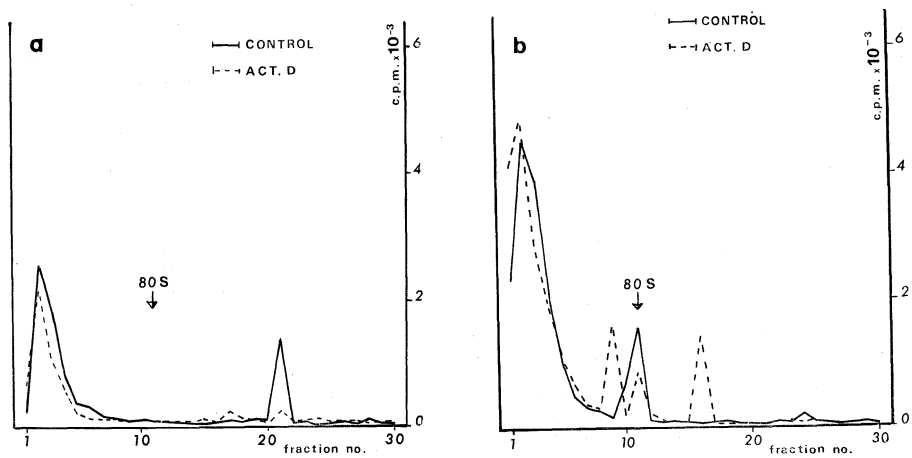

Fig. 3. – Sedimentation patterns of polysomes from the head region (*a*) and the dorsal region (*b*) of *Xenopus laevis* embryos cut and treated from early neurula stage on and separately incubated for 45 minutes at tail-bud stage.

In the patterns obtained from the ventral region of the embryos cut at the neurula stage and incubated for 18 hours, we found the same fractions which can be observed in the dorsal region (Fig. 4 a). In almost all the polysomal fractions the incorporation in the control is higher; fractions 18 and 26 are not lowered by the actinomycin.

Short incub at ionsat subsequent stages, as for the dorsal regions, were also carried out in the ventral regions. An incubation of 15 minutes at the neurula stage produces a very low incorporation both in the controls and in the actinomycin-treated embryos (Fig. 4 b); at this moment the activity of the endodermal cells is evidently very low. At the tail-bud stage (22 of N. and F.) some light polysomal fractions begin to be active, and these fractions are only slightly inhibited by the actinomycin; at this stage there can also be observed a heavy fraction which begins to become active and in which a certain superinduction can be noticed (Fig. 4 c). At the muscular response stage the activity of the light fractions increases and the embryos treated with actinomycin show a certain inhibition: on the contrary, the heaviest fractions remain superinduced (Fig. 4 d).

The ventral regions of the embryos cut at stage 22 and incubated for 18 hours show high activity in every polysomal fraction (Fig. 5). The incorporation

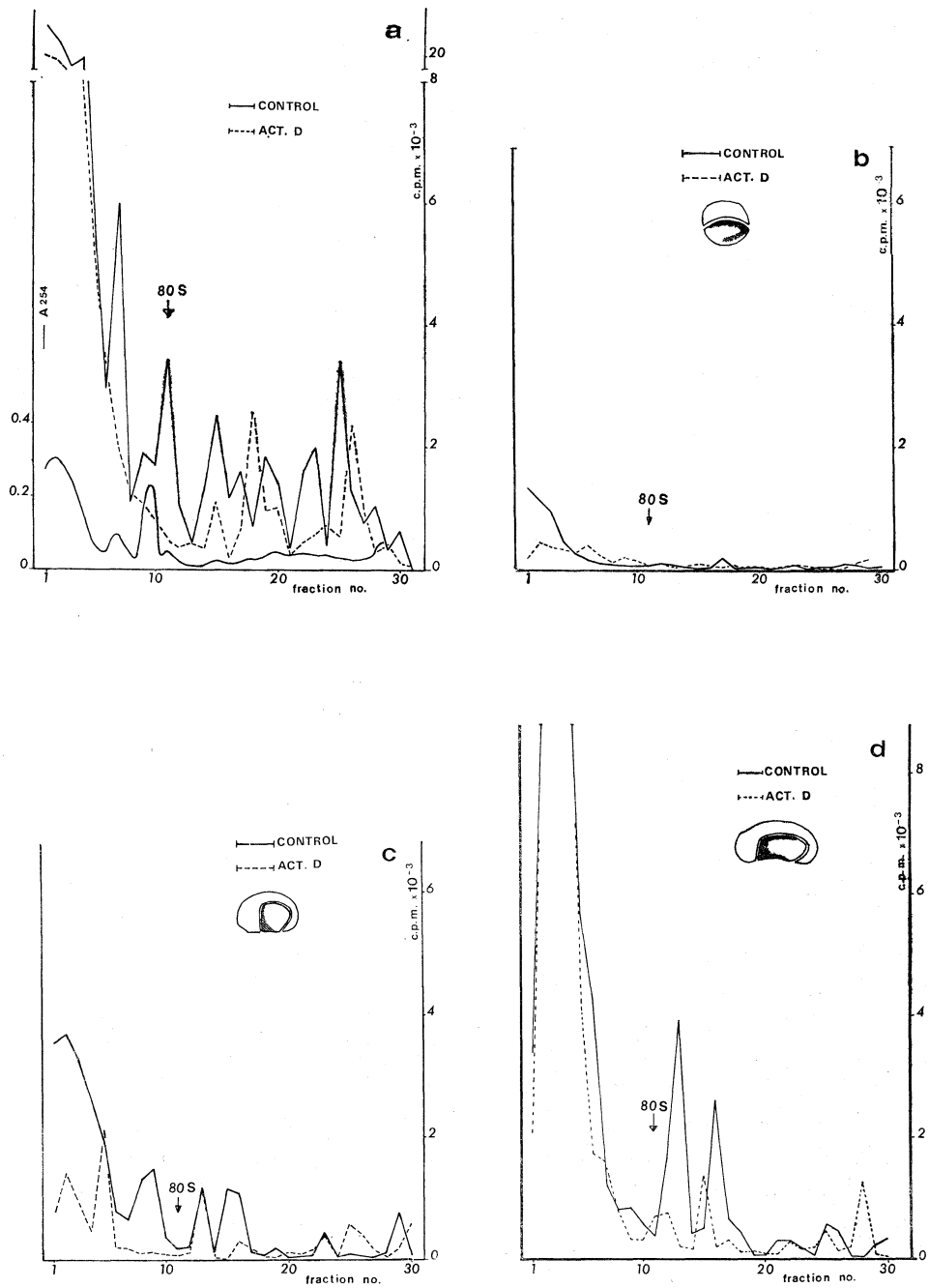

Fig. 4. – Sedimentation patterns of polysomes of ventral region of *Xenopus laevis* embryos cut at the early neurula stage (15 of N. and F.) and incubated with ^{14}C leucine for 18 hours (*a*) and 45 minutes (*b*, *c*, *d*), at the indicated stages.

is reduced in the treated embryos except in fraction 19, that is, on the contrary, superinduced, and in fraction 25 where the incorporation is almost the same as that observed in the control.

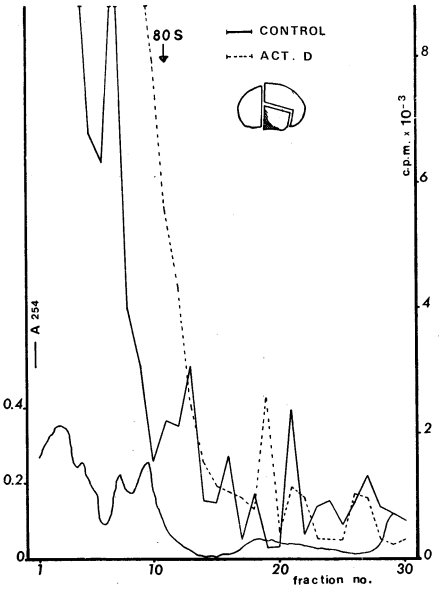

Fig. 5. – Sedimentation pattern of polysomes of the ventral region of *Xenopus laevis* embryos cut at tail bud stage (22 of N. and F.) and incubated for 18 hours with ^{14}C leucine.

Discussion. The inhibitory effect of Actinomycin D on RNA production has little influence on protein synthesis in the dorsal region of the *Xenopus* embryo during the closing of the neural tube. During this period protein synthesis seems to be supported by stable, long-lived messenger RNAs, which are synthetized between gastrulation and the beginning of the raising of neural folds. In a previous work (Leonardi Cigada *et al.*, 1975) we observed that leucine incorporation was reduced by 60 % in the dorsal region of the embryos cut at the early gastrula stage and treated with actinomycin. If the embryos were cut and treated at following stages (neural plate, early neurula) the inhibition by actinomycin was reduced.

If we compare the polysomal sedimentation patterns obtained after a long incubation (18 hours) with those obtained after a short incubation time, we notice that some polysomal classes subsequently begin to be active. This succession would correspond to a gradual activation of some mRNAs with the formation of polysomal complexes. In the dorsal region, between the neurula and tail-bud stages, the light polysomal fractions seem to be more active, while at the muscular response stage the greater incorporation is shown in the heavy fractions.

Some polysomal classes show a greater incorporation in the actinomycin-treated embryos than in the control. This paradoxical phenomenon was observed in eucaryotic cellular systems by several authors (Papaconstantinou,

1967; Moscona, 1968; Palmiter and Schimke, 1973; Leinwand and Ruddle, 1977) and differently interpretated. In our case the stimulation does not seem to influence indifferently the whole polysomal complex, but only some fractions of it, often those which are more active also in the control. In this case we cannot think of a mechanism affecting the translation machinery in its complex. Tomkins *et al.* (1969) collected quite a few data and elaborated a hypothesis for cellular systems subjected to hormonal induction; this hypothesis may fit in with the embryonic differentiation: in the fractions in which the radioactivity is higher in the treated than in the control protein synthesis is supported by mRNAs produced before actinomycin treatment (st. 15 for the embryos cut into two regions, st. 22 for the embryos cut into three regions). These mRNAs are controlled by some labile, fast turnover factors, perhaps of proteic nature, which are normally produced by the cells. When actinomycin inhibits the transcription, also the production of these repressors is stopped and the stable mRNAs can be translated without any regulation. Hence the apparent " superinductive " effect of the actinomycin. Tomkin's hypothesis seems to be suited to embryonic cells, those being subjected to inductive phenomena to a certain extent similar to those observed in the cell cultures.

The high activity of polysomes in the dorsal region during the closing of the neural tube corresponds to the differentiation steps of the nervous system and of the somites. This is a moment of active synthesis of a few kinds of specific proteins, generally codified by stable mRNAs (Palmiter and Schimke 1973; Gurdon *et al.*, 1974); these mRNAs may be present since very early developmental stages. Actually the highest superinduced peaks are observed in the dorsal region. If we agree with the previous hypothesis, we can suppose that these mRNAs, produced before the neurula stage, are controlled by repressors which are not produced in the presence of actinomycin. The result is the highest incorporation of the precursors in the polysomal fractions bound by these messenger RNAs.

The ventral region of the embryo shows a late histological differentiation (the differentiation of the endodermal organs begins more or less at stages 28–30). This fact can explain the very low activity of all polysomal classes at the neurula stage; evidently the cells synthesize only what is necessary for their own metabolism, without building anything new. Only at stage 22, which corresponds to tail bud formation, can we observe some kind of activity which becomes particularly intense from the muscular response stage on. In the ventral region we can see few fractions in which the incorporation is higher in the treated embryos than in the controls: generally the incorporation is lower in the treated embryos. This fact leads us to think that in the ventral region most mRNAs are immediately translated, on the contrary to what happens in the dorsal region. From the data of Woodland and Gurdon (1968) it seems that a great amount of " heterogeneous " RNA is synthesized in endodermal cells of the gastrula. From our results it seems unlikely that mRNA is stored in the endodermal cells till the tail bud stage; most probably the mRNA synthesized at early stages has a rapid turnover. We can suppose

that mRNAs coding for the differentiation of the endodermal organs are produced during the closing of the neural tube (st. 15–22) and are translated after the tail-bud stage.

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