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EFFECTS OF CHROMOMYCIN A 3 ON THE EMBRYONIC DEVELOPMENT OF *CIONA INTESTINALIS*

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A recent investigation of WAKISAKA *et al.* (1963) on mammalian cells, both normal and leukaemic, has shown that chromomycin A 3, an antitumoral agent extracted from *Steptomyces griseus*, inhibits selectively the biosynthesis of RNA, while the synthesis of DNA is not inhibited.

Actinomycin D is another substance which is well known as an inhibitor of the synthesis of the DNA-dependent RNA, and consequently the cells treated with this antibiotic can be considered as « chemically enucleated ».

The effects of this substance have now been established not only on bacteria and mammalian cells (DAVIDSON *et al.*, 1960; HURWITZ *et al.*, 1962; REICH *et al.*, 1962) but also on embryos; on the *sea urchins* by LALLIER (1963), GROSS & COUSINEAU (1963, 1964), MARKMAN (1963), MARKMAN & RUNNSTRÖM (1963); on the *amphibians* by BRACHET & DENIS (1963), FLICKINGER (1963), TOIVONEN *et al.* (1963), WALLACE & ELSDALE (1963); on *birds* by PIERRO (1961), KLEIN & PIERRO (1963).

In *sea urchin* eggs cleavage continues even at a concentration of actinomycin D of 100 γ/ml. Distortions in the segmentation planes occur, however; the migration of the primary mesenchyme cells into the blastocoele is delayed; gastrulation is blocked; the dorsoventral polarity of the larva differentiates precociously (MARKMAN, 1963; GROSS & COUSINEAU, 1963, 1964). According to MARKMAN (1963), the results agree with the view that a certain dorsoventral organization is already present in the unfertilized egg, but that a gene transcription is necessary after fertilization for establishing it.

In the eggs of *amphibians* (*Pleurodeles, Xenopus*) BRACHET & DENIS (1963) also noticed a normal segmentation. However, development is blocked at gastrulation (*Xenopus*) or at the neurula stage (*Pleurodeles*). At lower doses the nervous system (*Pleurodeles*) differentiates but the embryos are abnormal (microcephaly, absence of the eyes). On the basis of these results BRACHET & DENIS suggest that messenger RNA (mRNA) is not significantly produced before gastrulation. FLICKINGER's (1963) results obtained by exposing *Rana pipiens* embryos to actinomycin D for some days indicate that the synthesis of the various mRNA occurs only at the time of differentiation of the tissues. WALLACE & ELSDALE (1963) investigated the effects of actinomycin D on the embryos of *Rana pipiens*, and on their cells isolated *in vitro*. They obtained results which are similar to those of BRACHET & DENIS. Injections of high concentrations of actinomycin into the blastocoele of middle blastulae prevent gastrulation ; injections of lower concentrations produce exogastrulae or imperfect gastrulae which develop into poorly organized embryos ; injections of still lower concentrations (12.5 γ/ml) produce embryos arrested at tailbud stages, or normal larvae. The injections into the gastrocoele of the gastrulae allow a normal development. Cells isolated in heaps of 150-200, cultured in *vitro* with actinomycin (2 γ/ml), at first reaggregate normally but later lose adhesion. Morphogenetic movements of gastrulation and neurulation, as well as cellular differentiation, are very sensitive to actinomycin, while neural induction is less sensitive.

Chick embryos injected with 0.125 γ of actinomycin D into the yolk sac develop abnormally (« trunkless » or « rumpless ») according to the age of the embryo and the dosage employed. When embryos are cultured *in vitro* and treated with actinomycin, their embryonic axis posteriorly to the 12th somite is specifically inhibited (KLEIN & PIERRO, 1963). Protein nitrogen, DNA and RNA are present in lower amounts in the posterior region of the embryo. These results are interpreted by KLEIN & PIERRO admitting that the synthesis of the mRNA occurs only in the cells of the unsegmented somite mesoderm: the synthesis of mRNA in cells of segmented somite mesoderm, completed at earlier stages, would be immune to the action of actinomycin.

In the light of these findings it seemed desirable to conduct a detailed investigation on the action of other inhibitors of RNA synthesis on the developing eggs of the ascidians at various stages. The present paper reports the action of chromomycin A 3. The effects of actinomycin D, now being investigated in this laboratory, will be reported later.

MATERIAL AND METHODS

Eggs of *Ciona intestinalis* were obtained from freshly caught animals. Unfertilized and fertilized eggs at different stages of development were treated with chromomycin A 3 (Takeda Chemical Industries, Osaka, Japan). Table 1 indicates the concentrations (in sea water) of the substance, the stages and the periods of treatment. Only those results are presented for which the controls gave nearly 100% normal larvae. Treatment was performed in the dark, as is usually done with actinomycin, although nothing has been reported, to our knowledge, about the action of light on chromomycin A 3.

RESULTS

1. *Treatment of the unfertilized eggs (3 hours)*

Concentration of 5 γ/ml (2 experiments). The eggs were treated with chromomycin for 3 hours; afterwards they were carefully washed in sea water and then fertilized. These eggs segmented and developed in a perfectly normal way into normal, actively swimming tadpoles similar to the controls.

Concentration of 10 γ/ml (3 experiments). The treatment lasted for 5 hours; afterwards the eggs were washed and fertilized. They segmented and developed into normal, swimming tadpoles.

2. *Treatment of the fertilized eggs (3 hours)*

The eggs were treated with chromomycin at concentration of 10 γ/ml for 3 hours. In order to expose the unsegmented eggs to a longer period of treatment, in two experiments the eggs were treated at 10° C and then returned to sea water at room temperature. A few perfectly normal, swimming tadpoles developed. Control eggs, exposed to this « cold » treatment, developed mostly into abnormal larvae.

The treatment was repeated in two experiments at 14° C. At this temperature the controls developed normally. The eggs treated with chromomycin developed also into perfectly normal tadpoles.

These results show that chromomycin, at least at the concentrations indicated, does not affect the fertilized, unsegmented egg.

3. *Treatment from the 2-cell stage to hatching*

In these experiments eggs at the 2-cell stage were immersed in the solutions of chromomycin and allowed to develop there.

Concentration of 1 γ/ml (3 experiments). At this concentration chromomycin proved ineffective; the eggs always gave rise to normal tadpoles.

Concentration of 2 γ/ml (6 experiments). These eggs developed into larvae which possessed all their organs (palps, brain, sensorial organs, intestine, tail), but were morphologically and physiologically abnormal. In fact, the tails were short and curved; the movement was weak: consequently, the larvae were unable to swim and lay at the bottom of the culture dish. The effect exerted by the substance was evident in all larvae. Sometimes the sensorial organs (two pigmented spots) were also abnormal, being fused or reduced in size (Fig. 1 B).

Concentration of 3 γ or 5 γ/ml (6 experiments). The effects indicated above were more severe at this concentration. Often the larvae were not even able to hatch; even when they did, they were motionless. Their tails were reduced in length, and the notochordal cells irregularly arranged. The pigmented spots, brain and palps were missing. Therefore this concentration of chromomycin induces general abnormalities (Figs. 1 C, D).

The experiments reported above show clearly that chromomycin does not affect the unfertilized or just fertilized egg at concentrations of 10 γ/ml for 3-5 hours; at concentrations of 2-3 γ/ml, on the other hand, it induces strong abnormalities when the eggs are allowed to develop in the solution from the 2-cell stage. We thus obviously face the question: which stage of development is specifically sensitive to the action of chromomycin? In order to answer this question eggs were treated for 3 hours as follows: from cleavage to blastula stage; from blastula to mid gastrula; from mid gastrula to the end of gastrulation.

4. *Treatment from the 2-cell stage to blastula.*

Concentration of 3 γ/ml (3 experiments). The eggs developed in the normal way, giving rise to tadpoles which did not differ from the controls (Fig. 2 A).

Concentration of 4 γ/ml (1 experiment). Tadpoles mostly normal.

Concentration of 5 γ/ml (2 experiments). Normal tadpoles.

5. *Treatment from blastula to mid gastrula.*

Concentration of 3 γ/ml (3 experiments). The eggs developed in always normal, free swimming tadpoles (Fig. 2 B).

Concentration of 4 γ/ml (1 experiment). The results were not different: the larvae which developed were normal.

Concentration of 5 γ/ml (2 experiments). Normal tadpoles.

6. *Treatment from mid gastrula to the end of gastrulation.*

Concentration of 3 γ/ml (3 experiments). The effects of the substance were evident: the tadpoles lay on the bottom of the culture dish, as they were incapable of swimming, though their tails were moving; the tails were short and sometimes curved (Fig. 2 C).

Concentration of 5 γ/ml (2 experiments). The larvae lay on the bottom of the culture dish; they had a short, sometimes curved, tail, which scarcely moved. Some abnormalities in the brain and sensorial spots were also noticed.

These results show that mid gastrula to the end of gastrulation is the period of development most sensitive to chromomycin.

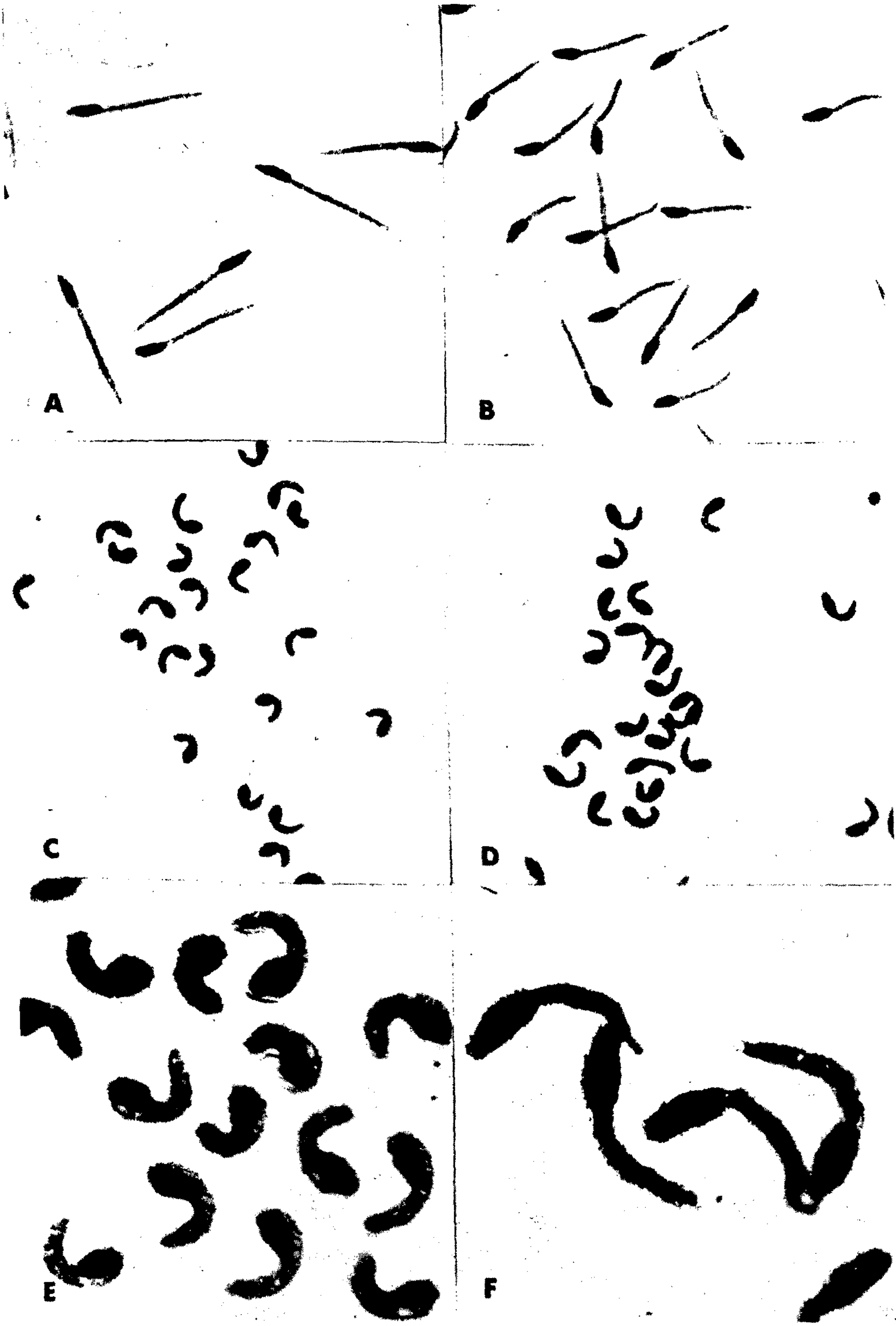

Fig. 1

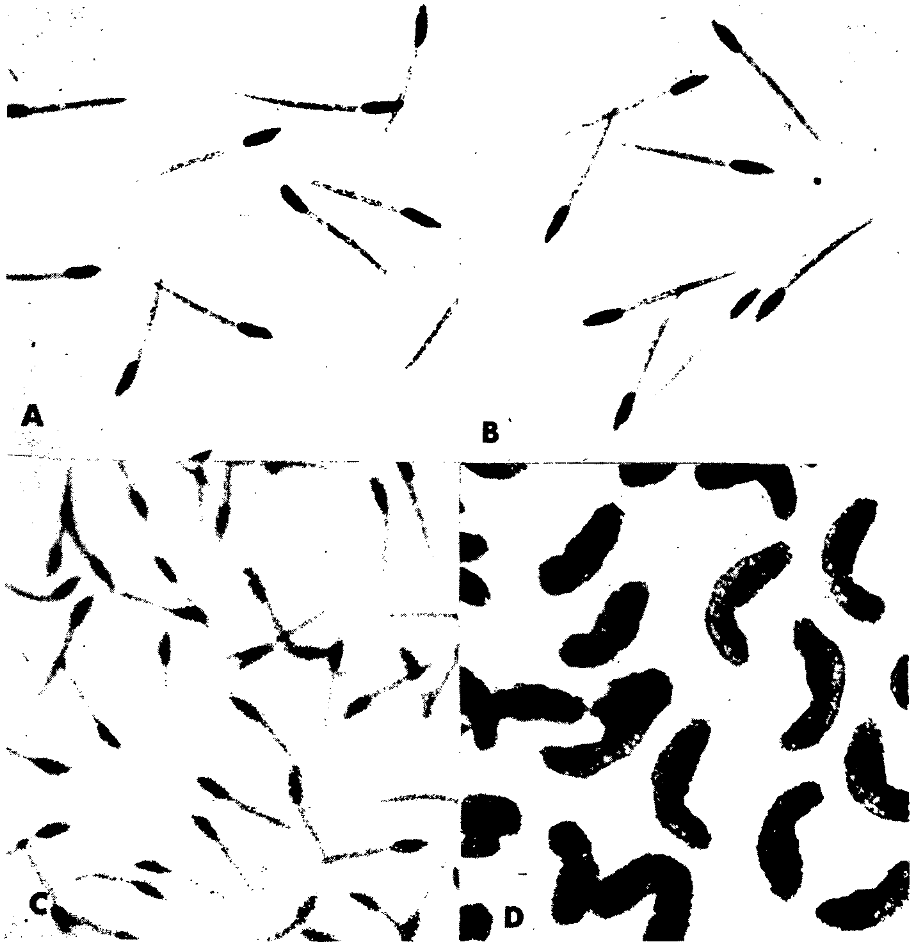

Fig. 2

Fig. 1. — *Ciona intestinalis* larvae.

A, controls. 30 ×.
B, from eggs treated with chromomycin (2 γ/ml) from the 2-cell stage up to the end of development. 30 ×.
C, from eggs treated with chromomycin (3 γ/ml) from 2-cell stage on. 30 ×.
D, from eggs treated with chromomycin (5 γ/ml) from 2-cell stage on. 30 ×.
E, higher magnification of the larvae represented in C. The abnormalities in the tail are evident ; the chordal cells are irregularly disposed ; the pigmented spots, the brain and the palps are absent. 90 ×.
F, higher magnification of the larvae reported in B. The tails are curved and shorter than in the controls. 90 ×.

Fig. 2. — *Cona intestinalis* larvae.

A, from eggs treated with chromomycin (3 γ/ml) from the 2-cell stage up to blastula stage. 30 ×.
B, from eggs treated with chromomycin (3 γ/ml) from blastula up to mid gastrula stage. 30 ×.
C, from eggs treated with chromomycin (3 γ/ml) from mid gastrula up to the end of gastrulation. 30 ×.
D, from eggs treated with chromomycin (10 γ/ml) for one hour, at the 7 th hour of development. The tails are much reduced and very short ; the chordal cells are disarranged. 90 ×.

TABLE 1

Effects of chromomycin A 3 on Ciona *development*

Stages of treatment	Concentr. γ/ml	Length of treatment	Temper. ° C	No. of exper.	Results
Unfertilized eggs	5	3 hrs.	18	2	+
	10	5 hrs.	18	3	+
Just fertil. eggs	10	3 hrs.	10	2	+
	10	3 hrs.	14	2	+
From 2-cell to hatching	1	24 hrs.	18	3	+
	2	24 hrs.	18	6	—
	3	24 hrs.	18	3	—
	5	24 hrs.	18	3	—
From 2-cell to blastula	3	3 hrs.	18	3	+
	4	3 hrs.	18	1	+
	5	3 hrs.	18	2	+
From blast. to mid gastr.	3	3 hrs.	18	3	+
	4	3 hrs.	18	1	+
	5	3 hrs.	18	2	+
From mid gastr. to end gastr.	3	3 hrs.	18	3	—
	4	3 hrs.	18	2	—
	5	3 hrs.	18	1	—
At 6 th hour of develop.	10	1 hr.	18	3	—
At 7 th hour of develop.	10	15 mins.		4	+
	10	1 hr.	18	3	—
At 8 th hour of develop.	10	1 hr.	18	3	—
At 9 th hour of develop.	10	1 hr.	18	3	—

+, normal development ; —, abnormal development (see text).

7. *Short treatments during gastrulation.*

To determine what phase of gastrulation was most sensitive to chromomycin, embryos that had developed for 6 hours in normal sea water and were at the mid gastrula stage (seventh hour of development) were treated with chromomycin at a high concentration (10 γ/ml) during four 15-minute intervals; afterwards they were returned to normal sea water where they developed to the larval stage. One part of the embryos was treated in the first 15 minutes; a second part in the *subsequent* 15 minutes, and likewise for a 3 rd and a 4 th part. The results showed that with such a short treatment the eggs developed in a normal way.

In a second experiment embryos were treated with chromomycin (10 γ/ml) for one hour during the 6 th, 7 th, 8 th or 9 th hour of development. The embryos developed into abnormal tadpoles. The most sensitive stages however, corresponded to the 7 th and 8 th hour of development, that is to the late stages of gastrulation (Fig. 2 D).

DISCUSSION

1. — The results obtained by this investigation can be summarized as follows:

(*a*) At concentrations of 5-10 γ/ml for 3-5 hours chromomycin does not exert any effect on the *unfertilized* egg.

(*b*) It does not exert any effect on the *fertilized,* uncleaved egg, either (10 γ/ml for 3 hours).

(*c*) The eggs which develop since the first cleavage in the solution of 1 γ/ml give rise to normal tadpoles. At concentrations of 2-3 γ/ml, however, they give rise to very abnormal tadpoles.

(*d*) At concentrations 3-5 γ/ml for 3 hours chromomycin does not exert much effect on the early stages of development (blastula and mid gastrula). It is, however, very effective on the *late gastrula.* Abnormal tadpoles are obtained with a treatment of 10 γ/ml for one hour. The more sensitive period corresponds to the 7 th and 8 th hours of development.

(*e*) The abnormalities which can be observed in the tadpoles are mostly limited to the *tail* and *sensorial organs.* The tails are short, curved and incapable of strong movements; as a consequence, the tadpoles are not able to swim and they lie on the bottom of the culture dish. The sensorial organs are also strongly affected; they are fused and/or reduced in size. The chordal cells are abnormally large.

(*f*) At high concentrations and for long periods of treatment, chromomycin induces abnormalities in all organs.

2. — The effects exerted by a certain substance on embryological development is always difficult to interpret. The embryo is a unity which is, however, constantly changing morphologically, physiologically and biochemically. Further, a substance, said to be specific in its effects, may well exert its action on more than one biological or biochemical system.

Remembering the above qualifying statements, we may try to drawn certain conclusions from the knowledge that chromomycin inhibits the biosynthesis of the DNA-dependent RNA, while allowing DNA biosynthesis to proceed. If we accept the explanation that chromomycin prevents a gene transcription or blocks the « delivery of informational RNA » our results are understood. Our results indicate that the period more sensitive to chromomycin is the late gastrula: at this stage the information would be produced and/or released to a marked degree. This interpretation is in agreement with our general knowledge concerning the metabolism of the gastrula: the end of the gastrulation process is characterised by an intense protein synthesis which accompanies the differentiation of embryonic territories.

3. — Short periods of treatment (one hour) at the end of gastrulation induce abnormalities in the tail and the brain, which are apparent in the larvae, i. e. at a later stage. This is comparable with FLICKINGER's (1963) finding, who suggests a sequential and not simultaneous release of specific mRNA. The treatment of frog eggs at the gastrula stage gave rise to motionless larvae having impaired muscle tissues, while there was a normal differentiation of sensory or digestive tissues. Similarly, in our study a short period of treatment leads to larvae with highly impaired tail musculature, while the brain is normal or less impaired. Longer periods of treatment lead to more general abnormalities, i. e. in all organs. Thus the « information » for normal tail differentiation is probably released during a short period in the course of gastrulation and chromomycin blocks this information. The same conclusion can be drawn for the differentiation of chordal cells, because with short periods of treatment these remain abnormally large or undifferentiated. The message for brain differentiation is perhaps released earlier or later, so that it is not blocked at the time of treatment. This proposed sequential release of specific mRNA for different organs and decline, if any, in the peak of RNA synthesis will be further investigated.

4. — Our results also suggest that in the unfertilized or just fertilized egg or in the embryo up to the mid gastrula stage the system of «information transfer» is not very active. The fertilized egg would already possess the amount of information which is necessary for differentiation up to the mid or late gastrula stage.

SUMMARY

1. The effects of chromomycin A 3 on the embryonic development of the ascidian *Ciona intestinalis* were investigated.

2. Chromomycin at concentrations of 5-10 γ/ml for 3 hours does not exert any effect on the unfertilized or fertilized, uncleaved egg.

3. The eggs which develop in chromomycin 2-5 γ/ml since the 2-cell stage give rise to abnormal larvae. The abnormalities are mostly limited to the musculature, chorda cells and sensorial organs. The larvae scarcely move and lie at the bottom of the culture dish.

4. The more sensitive period of development to the treatment with chromomycin extends from mid gastrula to the end of gastrulation (7 th and 8 th hour of development).

5. The results are explained in the frame of knowledge that chromomycin inhibits the synthesis of the DNA-dependent RNA.

RIASSUNTO

E' studiata l'azione della cromomicina A 3 sullo sviluppo embrionale di *Ciona intestinalis.*

La cromomicina a concentrazioni di 5-10 γ/ml per 3 ore non esercita alcun effetto dannoso sull'uovo vergine o sull'uovo fecondato e ancora indiviso. Essa esercita un effetto dannoso (alla concentrazione di 2-5 γ/ml) sulle uova che vi si sviluppano a partire dallo stadio di 2 blastomeri. Le larve che si ottengono da queste uova sono costantemente anomale.

Il periodo più sensibile all'azione della sostanza è quello compreso tra la gastrula media e la gastrula a termine (7^a e 8^a ora di sviluppo).

I risultati ottenuti sono interpretati in base alla conoscenza che la cromomicina blocca la sintesi dell'RNA dipendente dal DNA.

REFERENCES

Brachet J. & Denis H. — *Nature (London), 198,* 204 (1963).

Davidson E. H., Allfrey V. G. & Mirsky A. E. — *Proc. Natl. Acad. Sci. U. S., 49.* 53 (1963).

Denis H. — *Exptl. Cell Res., 30,* 613 (1963).

Flickinger A. — *Science, 141,* 1063 (1963).

Gross P. R. — *Proc. Natl. Acad. Sci. U. S., 51,* 407 (1964).

Gross P. R. & Cousineau G. — *Biochem. Biophys. Res. Comm., 10,* 321 (1963).

Gross P. R. & Cousineau G. — *Exptl. Cell Res., 33,* 368 (1964).

Hurwitz J., Furth J. J., Malamy M. & Alexander M. — *Proc. Natl. Acad. Sci U. S., 48,* 1222 (1962).

Klein N. W. & Pierro L. J. — *Science, 142,* 967 (1963).

Lallier R. — *Exper., 19,* 572 (1963).

Markman B. & Runnström J. — *Exptl. Cell Res., 31,* 615 (1963).

Pierro L. J. — *J. Exp. Zool., 147,* 203 (1961).

Reich E., Franklin R. M., Shatkin A. J. & Tatum E. L. — *Science, 134,* 556 (1961).

TOIVONEN S., VAINIO T. & SAXÉN L. — *Rev. suisse Zool., 71,* 139 (1964).
WAKISAKA G., UCHINO H., NAKAMURA T., SOTOBAYASHI H., SHIRAKAWA S., ADACHI A. & SAKURAI M. — *Nature (London), 198,* 385 (1963).
WALLACE H. & ELSDALE R. — *Acta Embryol. Morphol. Exper., 6,* 275 (1963).

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