The inhibitory effects of calf thymus-histone fractions on the development of the chick embryo

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The genetic potentialities of an egg are transcribed, translated and built up into a temporal-spatial organization by epigenetic processes. How are these potentialities realized? Efforts to answer this question have naturally turned towards a study of the activity of the genetic material, which according to modern ideas is entirely composed of DNA. Stedmann & Stedmann (1950) originally suggested that certain basic proteins (histones) present in the nucleus were gene inhibitors. Since then various biochemical aspects of histone metabolism have been studied (see review by Phillips, 1962). A number of distinct histones have been isolated (Butler, 1964). But tissues from different species of animals or different tissues from the same species have been found to possess very similar complements of histones (Hnilica, Johns & Butler, 1962). No specificity of interaction between histones and different parts of the DNA molecule has been observed (Johns & Butler, 1964). However, histones have been found to inhibit the synthesis of DNA-dependent RNA (Huang & Bonner, 1962; Barr & Butler, 1963; Allfrey, Littau & Mirsky, 1963; Bonner & Huang, 1964).

Changes in staining properties and distribution of DNA-associated basic proteins have been shown to accompany early developmental processes (Bloch, 1962; Bloch & Hew, 1960; Moore, 1963; Horn, 1962; Das, Kaufmann & Gay, 1964a, b). Such changes indicate a possible significant role of histones in differentiation. A study of the effects of extraneous histones on embryonic development could be expected to provide some evidence on this point. If histones are gene inhibitors, specific fractions might show inhibition of specific organ systems or at least a well-defined pattern of inhibition.

MATERIAL AND METHODS

The histone fractions used in these experiments, namely F1 (lysine-rich), F3 (arginine-rich) and F2a (intermediate) were obtained from calf thymus

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The fractions were dissolved in 0.9% sodium chloride solution and aliquots of 0.2 ml containing 0.5, 1 or 1.5 mg were injected into the albumen of unincubated hens' eggs as close to the embryonic disc as possible. After injection the eggs were left in the same position overnight and subsequently incubated for 48 h, at the end of which period the embryos were examined, fixed and sectioned. The controls were injected only with normal saline solution. Parallel experiments were performed with actinomycin D.

In another series (II) of experiments the treatment was carried out in vitro. Early chick embryos were explanted according to New's (1955) method. The histone solution (0.5 mg in 0.2 ml Pannett-Compton saline) was added to the embryos and 0.4 ml thin albumen outside the glass ring. Embryos at different stages of development (stages 3, 4, 5 and 8 according to Hamburger & Hamilton, 1951) were selected for treatment. Others at identical stages of development were maintained as controls. The control and treated embryos were incubated for 48 h. The in vitro experiments were intended primarily to study the effects on definite later stages of development and whether any change in pattern of inhibition was brought about by delaying the onset of treatment. They also helped to check the results obtained in series I experiments.

In order to find out whether the histones entered the cells of the embryo and where they were localized, some experiments were performed in which ³H-labelled F1 and F3 fractions (specific activity of F1, 40 mc/mg, and F3, 220 μc/mg), prepared by the Radiochemical Centre, Amersham, were used. The labelled fractions were diluted by unlabelled fractions and 120 μc of activity was introduced into the albumen of each egg. The embryos were sectioned at 6 μ. The slides were coated with Ilford K-5 nuclear research emulsion and exposed for 4–20 days. The autoradiographs were made according to the method of Kopriwa & Leblond (1962).

**EXPERIMENTAL RESULTS**

The three fractions of calf thymus histone (F1, F3 and F2a) used in these experiments produced similar effects on development (Table 1). Quantitative differences revealed in the table may not be meaningful, particularly because injection into albumen was not an ideal method owing to diffusion differences. In vitro experiments were performed only on F1 and F3, and effects of F2b were not studied in this way.

As is evident from Table 1 a large proportion (50–60%) of series-I embryos developed normally. This is probably due to dilution of the histone fraction in the egg albumen or may be due to imperfect diffusion as a result of which the histones did not reach the embryos. The autoradiographs of embryos injected with ³H-labelled histones which developed normally did not reveal any label, but those of the malformed ones showed a distribution of the label which was uniform throughout the embryo. No localization of the label was associated
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with any particular malformation. The label could be detected both in the cytoplasm and the nucleus (Table 2).

Table 1. Data on effects of histones (series I experiments)

<table>
<thead>
<tr>
<th>Histone fraction</th>
<th>No. injected</th>
<th>Normal (</th>
<th>Delayed development* (%)</th>
<th>St. 4–5 arrests (%)</th>
<th>Undifferentiated (%)</th>
<th>Abnormal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 C†</td>
<td>50</td>
<td>86</td>
<td>6</td>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>50</td>
<td>9</td>
<td>20</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>F3 C</td>
<td>45</td>
<td>81-4</td>
<td>4-4</td>
<td>6-6</td>
<td>2-2</td>
<td>4-4</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>65</td>
<td>55-4</td>
<td>7-7</td>
<td>21-5</td>
<td>4-6</td>
</tr>
<tr>
<td>F2a C</td>
<td>15</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>28</td>
<td>64-3</td>
<td>21-5</td>
<td>7-1</td>
<td>7-1</td>
</tr>
</tbody>
</table>

* Embryos around st. 8 were considered in this category. Development was normal but delayed compared to control embryos or normal treated embryos, which usually were at st. 11, 12 or 13.
† Control.
‡ Experimental.
§ One of these embryos also showed failure to form somites.

Three main effects were observed: (1) an arrest of development at the primitive-streak stage (st. 4) or early head process stage (st. 5), (2) malformation of the brain in embryos which developed beyond st. 5 in the presence of histones, and (3) a change in the pattern of malformation when onset of treatment was deferred to later stages of development.

Table 2. Grain counts in nucleus and cytoplasm

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Average grain count</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenchyme</td>
<td>23-7</td>
<td>48-1</td>
<td>0-49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoderm (pharynx)</td>
<td>15</td>
<td>22</td>
<td>0-68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Twenty-five mesenchymal and ten endoderm cells were counted. Background counts: 1-4/100 μ². Activity introduced 120 μc of ³H–F3 histone. Autoradiograph developed for 20 days.

1. Arrests of development

This was observed in 20–21 % of treated embryos, which remained at around st. 4. In some cases slight invagination of the head process was seen. The growth of blastoderms was as expected of st. 4–5 embryos. In some cases there was more growth. That this was not an extreme retardation in development was shown by its occurrence in st. 3–4 embryos which were cultured in vitro for 48 h in the presence of histones. In fact, arrest was observed in 80 % of these embryos. It can therefore be seen that the presence of histones caused arrest at st. 4–5
irrespective of when the treatment commenced. A section of an arrested primitive-streak embryo is given in Plate 1, fig. A.

Effects of actinomycin D were also similarly studied. High doses of the antibiotic (5 μg per egg) caused a complete cessation of development. The blastoderms showed no growth and resembled those obtained in unfertilized eggs. The range of dosage of 1-0-1 μg caused an arrest of development at st. 4–5. There was a gradation of effect with change of dosage. Higher dosage caused complete suppression of development in a greater number of cases (see Table 3). Actinomycin was more effective than the histone fractions in causing st. 4–5 arrest.

<table>
<thead>
<tr>
<th>No. injected</th>
<th>Normal (%)</th>
<th>Complete suppression (%)</th>
<th>St. 4–5 (%)</th>
<th>Brain malformed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23*</td>
<td>95.7</td>
<td>4.3</td>
<td>—</td>
</tr>
<tr>
<td>AD 5 μg</td>
<td>9</td>
<td>100</td>
<td>27.8</td>
<td>11.1</td>
</tr>
<tr>
<td>1 μg</td>
<td>18</td>
<td>61.1</td>
<td>22.2</td>
<td>—</td>
</tr>
<tr>
<td>0.5 μg</td>
<td>16</td>
<td>62.5</td>
<td>33.4</td>
<td>—</td>
</tr>
<tr>
<td>0.1 μg</td>
<td>9</td>
<td>22.2</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Only one egg was unfertile.

In some cases of histone-treated embryos a complete suppression of morphogenesis was observed and even the formation of the primitive streak seemed to have been suppressed, but there appeared dark lumps of undifferentiated cells in the centre of the area pellucida. Nevertheless, the blood islands formed normally and haemoglobin was synthesized. This was also observed in at least five cases of st. 4–5 arrest (see Plate 1, fig. B). It seems interesting that morpho-

**Plate 1**

Fig. A. Section through an embryo arrested at the primitive-streak stage.

Fig. B. This is an arrested st. 4 embryo of series I experiments. The sections showed an abundant invagination of the mesoderm. This embryo is illustrated to draw attention to the differentiation of blood islands and vessels. Usually the arrested embryos showed normal primitive-streak formation.

Fig. C. A control corresponding to arrested embryo in fig. B.

Fig. D. The forebrain is suppressed in this embryo (series I, F1, 1 mg/egg). The sections showed that the mid- and hind-brain were normal in shape. Lateral duplication of somites at the level of first and second pair; otherwise the somites, the neural tube and the heart were normal.

Fig. E. Control embryo for fig. D.

Fig. F. This is a section through forebrain of a series I embryo. The forebrain is suppressed and represented only by a mass of neural tissue with a tiny neural canal.

Figs. G, H. These embryos (series I, F1, 1 mg/egg) show disorganized brain. The brain of the embryo in fig. H was open throughout. They formed somites, heart and neural tube normally. See also Plate 2, fig. I.
genesis and formation of blood islands can be completely dissociated. The area vasculosa has been found to be metabolically active and to be able to synthesize haemoglobin even in the absence of the embryo (Hell, 1964b). Though de-embryonated the blastoderm in Hell’s experiments had no metabolic inhibitors acting on it. It is from this point of view that the present observations acquire importance. Obviously the histones have inhibited embryonic differentiation effectively, but allowed differentiation of blood islands and haemoglobin synthesis to continue. A similar phenomenon was reported in 1955 by Waddington, Feldman & Perry who found that chick blastoderms treated with the purine analogue 8-azaguanine showed inhibition of morphogenesis with the embryos remaining at st. 4 or 5 while in the area opaca and pellucida blood islands appeared.

Hell (1964b) studied the synthesis of haemoglobin in de-embryonated blastodiscs of 1-, 4-, 7-, and 10-somite-stage embryos treated with nucleic analogues such as bromodeoxyuridine (BUdR) and azaguanine, and actinomycin. Visible signs of haemoglobin synthesis appear at the 7-somite stage. BUdR was found to inhibit haemoglobin synthesis if treatment commenced at 1- or 4-somite stage but there was no inhibition if it commenced at 7- or 10-somite stage. Azaguanine was more effective before or up to the 4-somite stage than at 7- or 10-somite stages. Actinomycin D at high concentrations completely inhibited haemoglobin synthesis, while at lower concentrations the inhibition was about 40 % in all stages. The results have been interpreted by Hell as indicating that messenger RNA for haemoglobin is synthesized before 1-somite stage.

The initiation of haemoglobin synthesis following application of several analogues and antibiotics has also been studied by Wilt (1965). His results seem to suggest that the synthesis can be inhibited if RNA metabolism is interfered with about 9 h before onset of haemoglobin synthesis.

### Plate 2

Fig. I. This embryo (series I, F1, 1 mg/egg) shows disorganized brain. It formed somites, heart and neural tube normally. See also Plate 1, figs. G, H.

Figs. J, K. Embryo treated with 1.0 mg F1/egg (series I). The brain was throughout open and plate-like. There was degeneration which, however, was confined to the brain (see fig. K).

Fig. L. An embryo treated with F1 fraction at the late head process stage. The brain is formed. The heart is also formed but pushed towards the anterior. The somitic axis is completely suppressed.

Fig. M. Embryo at late-head-process stage treated with F1 fraction. Note the supernumerary somites.

Fig. N. Section passing through supernumerary somites. Note the neural plate.

Figs. O, P. Stage-8 embryos treated with F1. Note the irregular somite formation beginning at the arrow.

Fig. Q. Control embryo corresponding to figs. O and P.
2. Malformation

Embryos which developed beyond st. 4–5 in the presence of histones showed brain malformations, which consisted of suppression (Plate 1, figs. D, F) or disorganization (Plate 1, figs. G, H; Plate 2, fig. I). In a large number of cases the brain was partly or completely open and plate-like (Plate 1, fig. H; Plate 2, figs. J, K). Degeneration of the brain tissue was observed in several cases and was confined to the brain tissue (Plate 2, fig. K). Brain malformation occurred if treatment was begun any time during formation of the primitive-streak and occasionally when st. 4–5 embryos were treated (Table 4).

Table 4. Effects of histones on chick embryos in vitro

<table>
<thead>
<tr>
<th>Stage treated</th>
<th>No. treated</th>
<th>Normal (%)</th>
<th>Abnormals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arrest</td>
</tr>
<tr>
<td>3–4</td>
<td>C 10</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>E 10</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>C 10</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>E 10</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>C 16</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>E 30*</td>
<td>20</td>
<td>—</td>
</tr>
</tbody>
</table>

* Includes Fl- and F3-treated. Stages 3, 4 and 5 were treated with Fl only.

3. Change in pattern of inhibition

When embryos at late st. 5 (late head process) or older were treated with histones in vitro the brain seemed to develop reasonably normally whereas the somitic axis was affected (Table 4). Usually there occurred a suppression or a shortening of the somitic axis (Plate 2, fig. L) or there was a formation of supernumerary somites occasionally arranged like a strip of mosaic on both sides of the neural tube (Plate 2, figs. M, N). In some cases the mesodermal bands were unsegmented. The neural tubes were usually open and plate-like or wavy (Plate 2, fig. N). A number of 4- to 5-somite embryos were treated and it was observed that the somites added after the treatment began were affected (Plate 2, figs. O, P). Study of sections of the embryos showed that other organs such as the notochord, pharynx, heart, etc., formed normally. Only one instance of notochordal abnormality was recorded and it may not be significant.

It is very clear that the pattern of inhibition changes if treatment is postponed to later stages of development.

DISCUSSION

The present study indicates that histones can modify the processes of development and the modifications produced have some characteristic features. The experiments with labelled histones show that the effects observed were due to the presence of histones. It is not certain in what form they were effective as they
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may not enter the cell in the form in which they are introduced. They are also liable to be degraded by enzymes present at the cell surface.

Two of the main effects described here, namely, arrest of development at st. 4–5 and malformation of brain, have already been reported in studies on the Amphibia. Markert & Ursprung (1963) and Kimmel (1964) obtained arrest of development of amphibian embryos at the late blastula stage by injecting cytoplasmic and nuclear protein fractions prepared from adult frog liver. Histones were one of the fractions studied. It is interesting that they noted a significant increase in the capacity of isolated nuclear histones to cause blastular arrest as compared to the nucleohistone complex. Brachet (1964) has also reported arrest of development at the late blastula stage as a result of injection of histone fractions. Embryos at late blastula or early gastrula were sensitive to the presence of histones in the medium, but late gastrulae were insensitive. When treated embryos were returned to normal medium, developmental abnormalities like microcephaly, cyclopia, a complete absence of the nervous system and spina bifida occurred.

Apart from the effects described, a point of particular interest is the similarity between the effects of histones and actinomycin D. The latter caused st. 4–5 arrest in the present experiments. Developmental arrest during gastrulation as a result of actinomycin treatment has been reported in certain species of Amphibia (Flickinger, 1963; Brachet & Denis, 1963). Actinomycin was also found to cause various degrees of brain malformation, lack of differentiation and degeneration of the system (Flickinger, 1963; Brachet & Denis, 1963; Brachet, Denis & de Vitry, 1964). According to Brachet & Denis (1963) effects on chick embryos were similar to those on amphibian embryos, the brain being the principal organ affected by the treatment. It was also reported that the malformation caused depended upon when the treatment began (Flickinger, 1963).

The results of the present series of experiments do not seem to support the view that the histones are the specific gene inhibitors, the removal of which determines morphogenesis. If they were, it should have been possible to associate the origin of different tissues with different histones. In biological experiments such as have been reported here it should have been possible to bring about inhibition of specific organ systems with specific histone fractions provided that they represented distinct biologically active entities. The well-defined pattern of inhibition produced by histones, the fact that different histone fractions produce similar effects and the similarity of the effects of the latter and actinomycin, however, seem to suggest that added histones have a more general type of effect. A mechanism by which they might have altered the processes of development is suggested below.

It is an established fact that actinomycin inhibits the synthesis of DNA-dependent RNA (Reich, 1964; Goldberg & Reich, 1964). It has been shown that histones also inhibit DNA-dependent RNA synthesis (Huang & Bonner, 1962; Barr & Butler, 1963; Allfrey et al. 1963). It might be thought the similarity in
the effects is because both inhibit the synthesis of DNA-dependent RNA, but the mechanism of their action is probably different (see later discussion). Viewed in this light, the arrest of development at st. 4–5 (considered homologous to gastrulation in the Amphibia) would indicate that the processes of differentiation have come to a stop because of an inhibition of flow of genetic information. An important phase of genetic transcription thus seems to begin at st. 4–5. This was suggested earlier by Brachet and his colleagues in experiments on the Amphibia (Brachet, 1965). Recent investigations indicate that messenger RNAs may be present, preformed in or synthesized during the cleavage phase of development. These messengers are probably concerned with the synthesis of enzyme systems and co-factors essential for synthesizing proteins needed for cleavage divisions or those characterizing differentiation (see Sherbet, 1966, for a detailed discussion).

It could be argued, therefore, that the regulation of the synthesis of messengers might be one of the controlling factors in development. When one interferes with such synthesis one encounters certain developmental abnormalities.

If treatment is commenced sufficiently early both actinomycin and histones produce brain abnormalities. It is difficult to believe that both of them inhibit specifically the synthesis of those messenger RNAs which are concerned in the differentiation of the nervous system. What seems more likely is that their effect is non-specific and consists in altering the overall rate of RNA synthesis. It would be recalled here that the purine analogues, azaguanine and benzimidazole, which interfere with synthesis of RNA, have produced severe effects on brain formation in chick embryos. These included retardation in its development, open and flat brains, and an excessive degeneration of the system (Waddington et al. 1955; Waddington & Perry, 1958). The first signs of axial formation in embryogenesis is the formation of the nervous system in response to the primary embryonic induction. It is known that neural competence is lost early in development (Waddington, 1940). With the reduced rate of RNA synthesis the optimal level is not reached and this results in neural differentiation in only small masses of the tissue before loss of its competence is complete.

The change of pattern of inhibition with postponement of treatment cannot be explained away by merely suggesting that synthesis of the messenger RNAs concerned is sequential and that only those species of RNA being formed at time of treatment are interfered with. For, in the embryos treated early, despite the presence of histones in the system at the beginning of somite formation, the somites formed normally. The possibility that the histones were destroyed by proteolytic enzymes before the processes of somite formation began could be reasonably excluded because they were present in the medium throughout the duration of the experiment. If we were to presume that a simultaneous production of all the messengers concerned occurred at, say, gastrulation and that a decrease in rate was brought about by histones, the embryos which received the histones later in development should still show normal somite formation.
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The situation could be explained on the basis that the experimentally introduced histones act not directly on the DNA but on substances in the cytoplasm concerned indirectly with gene activity (plasmotropic action, cf. Waddington, 1962). This is compatible with the recent observations of Gurdon & Brown (1965) that the type of RNA synthesized in the nucleus is dependent on the kind of cytoplasm in which it is located. If a nucleus which synthesizes ribosomal RNA from a neural-fold-stage embryo is transplanted into an enucleated egg it stops further synthesis of ribosomal RNA. Similarly blastula nuclei which are about to start synthesis of ribosomal RNA do not do so when put into enucleated eggs, but synthesis starts at onset of gastrulation as in normal embryos. Thus the state of derepression of genes is easily reversible and is dependent upon factors which are present in the egg.

It has been also shown that a two-directional migration of protein between nucleus and cytoplasm occurs (Goldstein, 1958). One could suggest from these data that some macromolecules which are concerned in the regulation of gene activity pass into the cytoplasm where they are modified and go back to the genes and derepress them. Which genes they would derepress depends upon the molecules in the cytoplasm with which they interact.

A possible mechanism of histone action

An important presumption is made here. It is that histones are concerned in gene inhibition though they may not be the exclusive gene inhibitors. The regulator genes produce allosteric protein molecules. These molecules combine with the effectors by the allosteric site and this results in the establishment of reactive sites on the allosteric proteins suitable for binding the histones present on the DNA. But the experimentally introduced histones complex with the modified allosteric molecules as soon as they are modified and as a result the genes remain repressed.

As already stated the neural induction is the first phenomenon and the effectors are present in the cells at the time of the induction, possibly combined with allosteric regulator gene products. The differentiation of somites or other systems is a subsequent process, though possibly some temporal overlapping might be present. It is reasonable to expect that the effectors concerned with somite formation are fewer in number compared to the effector molecules concerned in the induction of the nervous system. In other words, there appears a competition between the two for the extraneous histones and naturally the latter will complex with the more numerous neural effector-allosteric complexes. However, if the introduction of the histones is delayed until the process of induction of the brain is over there is no competition from neural effector allosteric complexes. On the contrary there are comparatively more somitic effector complexes with which the histones could combine and cause a change in the inhibition pattern.
SUMMARY

The effects of F1, F3 and F2a fractions of calf thymus histone on the development of chick embryos have been studied. The main effects which are produced by all three fractions are as follows: (1) There is an arrest of development at the primitive-streak stage (st. 4) or early head process stage (st. 5). (2) The embryos which developed beyond st. 4–5 showed suppressed, disorganized, completely or partly open and plate-like brain formation. When degeneration occurred it was confined to the brain tissue. (3) If treatment was commenced at later stages of development the inhibition shifted to the somitic axis which was suppressed, shortened or showed lack of segmentation of somitic mesoderm or formation of supernumerary somites. The effects are strikingly similar to those produced by actinomycin D.

The significance of these observations is discussed in relation to the role of gene regulation suggested for the histones.

RESUMÉ

Les effets inhibiteurs de fractions d'histone de thymus de veau sur le développement de l'embryon de poulet

Les effets des fractions F1, F3 et F2a des histones de thymus de veau sur le développement de l'embryon de poulet ont été étudiés. Les effets principaux de ces 3 fractions sont les suivants: (1) un arrêt du développement au stade de la ligne primitive (st. 4) ou du jeune processus céphalique (st. 5). (2) Les embryons qui se développayaient au delà du st. 4 ou 5, avaient leur cerveau supprimé, désorganisé, complètement ou partiellement ouvert et plat. Lorsqu’il y avait une dégénérescence, elle se limitait au tissu cérébral. (3) Si le traitement était appliqué à des stades plus tardifs, l’inhibition affectait l’axe des somites qui était alors supprimé, raccourci ou bien encore le mesoderme ne se segmentait plus en somites ou au contraire donnait des somites surnuméraires. Ces effets ressemblent de façon frappante à ceux produits avec l’actinomycine D.

La signification de ces observations est discutée compte tenu du rôle de régulateur génétique suggéré pour les histones.

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