sensitivity to cold inactivation in this medium. Probably the high content of protein (SH-groups) in serum exerts a protective influence on the enzyme molecules. Nevertheless, LDH activity in serum is better preserved when kept at room temperature instead of in the refrigerator, especially in the case of a high concentration of LDH-5.

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9 September 1963

Actinomycin D: Specific Inhibitory Effects on the Explanted Chick Embryo

Abstract. *Chick embryos containing 11 to 13 somites were cultured for 48 hours on media containing various amounts of the antibiotic actinomycin D. Morphological observations as well as quantitative determinations of protein nitrogen, DNA, and RNA indicate that the antibiotic specifically inhibits the growth and development of the embryonic axis posterior to the 12th somite.*

Injection of 0.125 μg of the antibiotic actinomycin D into the yolk sac of chick embryos during the second day of incubation causes cell death in the undifferentiated nodal mass and in the unsegmented somite mesoderm (*1*). Subsequent degeneration in these regions interferes with normal development of the embryonic axis (*2*). Depending on the extent of interference, abnormal embryos so produced have been classified as either "trunkless" or "rumpless." Trunkless embryos are (i) those which consist solely of a head, neck, and heart region, (ii) those which lack portions of the trunk, including the hind appendages, or (iii) those which have constricted trunks and abnormal limbs. Rumpless embryos lack the pygostyle, free caudal vertebrae, and, usually, varying numbers of the synsacral vertebrae. The extent of the axial defect resulting from treatment with the antibiotic is related to the developmental stage of the embryo at the time of treatment as well as to the dosage employed. Trunklessness is more common after treatment at 32 hours' incubation with 0.0625 μg of the antibiotic, and rumplessness is more common after treatment at 48 hours' incubation. The frequency of trunklessness obtained after treatment at 48 hours can be increased, however, by increasing the dosage of antibiotic.

We have undertaken to elucidate the mechanism of the deleterious action of actinomycin D in the chick embryo. Because there is considerable variation in developmental stages among embryos of any given incubation age, and because the response of an embryo to the antibiotic varies with the developmental stage, we are using chick embryos cultured in vitro. The in vitro explantation technique allows for standardization of the experimental material at specific developmental stages, assures uniform distribution in the medium of the substance to be tested, and facilitates use of quantitative procedures for analyzing the response. Having completed the preliminary phases of these studies, we now present evidence inditating (i) that actinomycin D interferes specifically with the development of the axial skeleton of the chick embryo cultured in vitro and (ii) that this interference is restricted to those levels of the embryonic axis which are normally elaborated from the unsegmented somite mesoderm and undifferentiated nodal mass subsequent to the time the embryos are exposed to the antibiotic.

Fertile White Leghorn eggs from a local commercial source were incubated in a forced-draft incubator at 37.5°C for approximately 40 hours, and embryos containing 11 to 13 somites were selected for explantation according to the procedures of Klein *et al.* (*3*). Embryos were explanted onto a concentrated whole egg homogenate medium (*4*) to which various amounts of actinomycin D had previously been added. Control embryos were cultivated on the medium alone. For the first 24 hours, all cultures were maintained under an atmosphere of 25 percent oxygen and 75 percent air. For the next 24 hours the atmosphere was changed to 95 percent oxygen and 5 percent carbon dioxide (*5*).

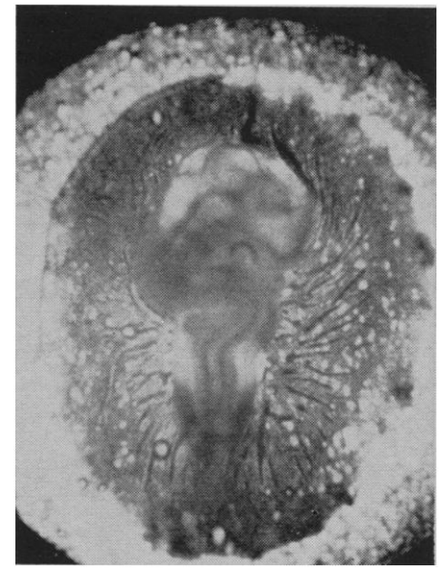

Fig. 1. Control embryo explanted when it had 11 to 13 somites and then cultured for 48 hours in vitro.

Under these conditions 57 of 58 control embryos survived 48 hours and showed well-developed heads, tails, and limb buds (Fig. 1). All embryos (55) exposed to 0.125 μg of actinomycin D, 52 of 57 embryos exposed to 0.25 μg, and 28 of 57 embryos exposed to 0.5 μg survived the culture period. The response to 0.125 μg of the antibiotic was variable, but virtually all embryos exposed to higher doses showed some

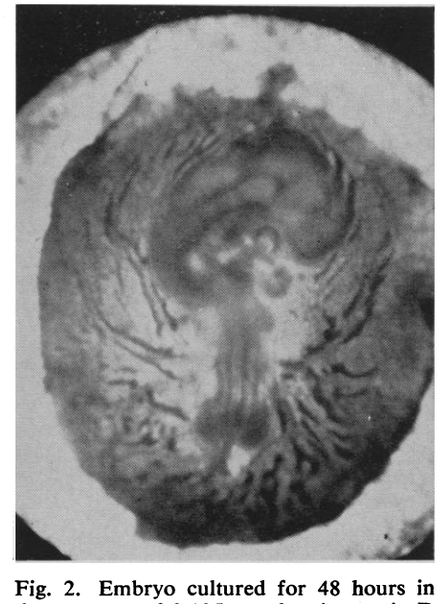

Fig. 2. Embryo cultured for 48 hours in the presence of 0.125 μg of actinomycin D per milliliter.

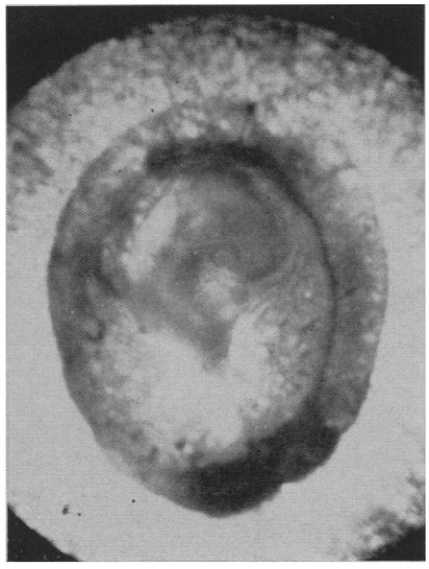

Fig. 3. Embryo cultured for 48 hours in the presence of 0.5 μg of actinomycin D per milliliter.

disturbances of axial development. Abnormalities included reduction in the thickness and length of the tail (Fig. 2), absence of a tail, and absence of various portions of the embryonic axis posterior to the 12th somite (Fig. 3). The head regions of the embryo did not appear to be affected by exposure to the quantities of actinomycin D used, a finding that is in agreement with previous reports (*1, 2*).

Quantitative analysis of the response of the explanted chick embryos to treatment with actinomycin D was carried out on tissues combined at random from three explants of the same experimental group. Embryos were trimmed of their extra-embryonic membranes according to the procedure of Hayashi and Herrmann (*6*) and then were divided into two portions by a cut between the 12th and 13th somites. Since embryos containing 11 to 13 somites had been selected for explantation, we expected that a cut behind the 12th somite would, on the average, separate both the head and the axial material including the somite mesoderm already segmented at the time of explantation (anterior fraction) from the axial material derived from the tail bud and somite mesoderm unsegmented at the time of explantation (posterior fraction). Protein nitrogens (PN) were then determined by Nesslerization according to the procedure of Britt and Herrmann (*4*), DNA by the method of Schneider (*7*), and RNA by the phloroglucinol method (*8*).

Data summarized in Table 1 showed that accumulation of PN, DNA, and RNA in the anterior portion of the embryo was not affected even at the highest dose of antibiotic. Values for PN and RNA of the anterior portion were somewhat lower for the group of embryos exposed to 0.5 μg of antibiotic than values for control embryos, but the differences were not statistically significant ($P > .1$). On the other hand, values for PN, DNA, and RNA were all significantly reduced ($P < .01$) in the posterior fractions of the embryos exposed to 0.25 and 0.5 μg of antibiotic. Even with the lowest dosage of actinomycin D used, values for PN, DNA, and RNA were lower than in the controls, but the differences were not significant. In a number of cases, the accumulation of PN, DNA, and RNA was also determined for the materials trimmed away from the embryonic axis, mainly the extra-embryonic yolk-sac membranes. As Table 1 shows, no significant differences in any of these parameters were observed between the membranes of the control and those of any of the experimental group.

The lower values for PN, DNA, and RNA obtained on analysis of the posterior regions of the treated embryos are expected in view of the gross observations. What is more significant is the demonstration that similar amounts of PN, DNA, and RNA are accumulated in the anterior embryonic regions of both the control and treated embryos. These data strongly reinforce the morphological observations because they show a specific regional response of the chick embryo to treatment with actinomycin D. Clearly, development of the head and portions of the trunk derived from somite mesoderm already segmented at the time of explantation is not affected by quantities of actinomycin D which effectively interfere with development of the unsegmented somite mesoderm and more posterior embryonic regions. In view of the earlier studies which indicated that more posterior regions of the embryonic axis become defective with an increase in developmental age (*1, 2*), it seems apparent that the susceptibility of the cells of the undifferentiated nodal mass towards actinomycin D is modified as the cells are modeled into the nerve cord, notochord, and gut. Similarly, the segmentation of the somite mesoderm appears to be accompanied by cellular modifications which provide for the acquisition of some degree of resistance to actinomycin D.

Considerable evidence is available which indicates that actinomycin D interferes with the synthesis of DNA-dependent RNA in a wide variety of systems, both cellular and cell free (*9*). If a similar mechanism of action is responsible for the deleterious effects of actinomycin D on the developing chick embryo, the regional specificity of the response suggests, as one possibility, that in chick embryos with 11 to 13 somites DNA-dependent RNA synthesis occurs mainly in the cells of the unsegmented somite mesoderm and the nodal mass. Since the RNA involved is presumably the so-called messenger-RNA (*10*), this explanation suggests as a corollary that the synthesis of messenger-RNA is completed before the prospective nerve and notochord cells

Table 1. Protein nitrogen (PN), DNA, and RNA of various regions of embryos explanted for 48 hours on media containing various amounts of actinomycin D. Means and standard errors are shown. The numerals in parentheses show the number of determinations (tissue from three explants made one determination). The anterior fraction consisted of head and axial material through the first 12 somites. The posterior fraction consisted of axial material posterior of the 12th somite. The membranes consisted of yolk sac, limb buds, and lateral wall.

Dosage (μg/ml = μg per embryo)	Accumulation (μg per embryo)		
	PN	DNA	RNA
	Anterior fraction		
0	38.5 $\pm$ 1.5(16)	21.4 $\pm$ 1.1(16)	38.1 $\pm$ 2.3(8)
0.125	40.0 $\pm$ 1.4(18)	22.9 $\pm$ 1.0(17)	38.8 $\pm$ 2.5(9)
0.25	39.7 $\pm$ 1.5(15)	21.9 $\pm$ 1.1(16)	37.2 $\pm$ 1.5(7)
0.50	33.8 $\pm$ 2.6(7)	18.3 $\pm$ 1.7(7)	32.9 $\pm$ 0.2(2)
	Posterior fraction		
0	8.6 $\pm$ 0.2(16)	6.7 $\pm$ 0.3(16)	8.9 $\pm$ 1.4(8)
0.125	7.1 $\pm$ 0.4(18)	5.9 $\pm$ 0.3(17)	6.1 $\pm$ 1.2(9)
0.25	5.3 $\pm$ 0.4(15)	5.1 $\pm$ 0.3(16)	3.2 $\pm$ 1.3(7)
0.50	2.4 $\pm$ 0.2(7)	3.5 $\pm$ 0.4(7)	$<$ 2.0(2)
	Membranes		
0	78.5 $\pm$ 4.8(8)	25.9 $\pm$ 2.7(8)	55.6 $\pm$ 7.5(8)
0.125	87.1 $\pm$ 5.7(8)	27.2 $\pm$ 2.4(8)	54.2 $\pm$ 4.9(8)
0.25	79.8 $\pm$ 6.9(6)	24.0 $\pm$ 1.9(6)	49.6 $\pm$ 4.3(6)
0.50	70.3 $\pm$ 6.5(2)	18.4 $\pm$ 2.0(2)	37.2 $\pm$ 4.9(2)

leave the undifferentiated nodal mass. Similarly, it is possible that synthesis of messenger-RNA in cells of the somite mesoderm is completed before segmentation. That a relatively stable messenger-RNA would have to be synthesized in the resistant cells seems to follow as a necessary consequence of these considerations. Such a suggestion appears feasible in view of the studies indicating that the stability of messenger-RNA varies in different cell systems (*11*). Alternative explanations for the regional specificity of the response of chick embryos with 11 to 13 somites to treatment with actinomycin D are also possible. Serious consideration should be given, for example, to the possibility of differential permeability to the antibiotic. Other investigators (*12*) have shown that, although *Escherichia coli* is resistant to actinomycin D, DNA-dependent RNA synthesis can be inhibited in protoplasts prepared from *E. coli*. The suggestion that the resistance of intact *E. coli* to actinomycin D may be related to the impermeability of the organism to this antibiotic is readily apparent (*13*).

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Provitamin A_2 from Lutein

Abstract. *Alfalfa lipids were treated with* p-*toluenesulfonic acid in benzene under reflux; after saponification, chromatography of the unsaponifiable portion yielded a carotenoid having a single absorption maximum at 460 mμ in ethanol and hexane. Its properties correspond to those of the dehydration product of lutein, 3′-hydroxy-3,4-dehydro-β-carotene, for which the name "anhydrolutein" is proposed. Vitamin A depleted chicks convert this pigment to vitamin* A_2*, as shown by the high ratio of absorbancies at 693 and 620 mμ in a mixture of* $CHCl_3$ *and* $SbCl_3$ *and the absorption maximum at 350 mμ in hexane exhibited by the liver lipids. The possible role of this pigment in the biogenesis of vitamin* A_2 *is discussed.*

The predominance of vitamin A_2 over vitamin A_1 in fresh-water fish and some amphibians presents a puzzle to the biochemist. Whereas a number of carotenoids have been shown to exhibit provitamin A_1 activity in many animal species, no such precursors are known for vitamin A_2, the origin of which remains obscure. According to one report, β-carotene caused an increase in the amount of both vitamins A_1 and A_2 in certain fresh-water fishes (*1*), but the significance of this observation with regard to the biogenesis of vitamin A_2 is not clear.

The structural difference between the two A vitamins, and the fact that they are not interconvertible in vivo, suggest that a possible requirement for provitamin A_2 activity might be the presence of an unsubstituted 3,4-dehydro-β-ionone ring in the carotenoid molecule. Compounds of this type would be expected to be converted to vitamin A_2 in animals by the same series of reactions that lead to the formation of vitamin A_1 from its precursors. Furthermore, vitamin A_2 formation should then be possible in all those species in which vitamin A_1 is produced.

Several derivatives of 3,4-dehydro-β-carotene fulfilling the above structural condition were prepared by Zechmeister and his co-workers and supported growth in the vitamin A deficient rat (*2*, *3*). The most interesting of these compounds from the point of view of the biogenesis of vitamin A_2 is 3′ hydroxy-3,4-dehydro-β-carotene or "deoxylutein I" (*3*, *4*), which is formed from lutein by the loss of one molecule of water from the α-ionone ring, a reaction which might conceivably occur in nature. Zechmeister and Sease (*4*) reported yields of 3 to 4 percent provitamin, based on lutein, by melting a mixture of lutein, naphthalene, and boric acid (or tetraboric acid or boric anhydride). We have recently encountered the pigment in acidulated soybean soapstock (*5*) which had previously been reported to enhance the liver storage of vitamin A in chicks (*6*). Its presence in the soapstock was shown to result from the action of mineral acid on lutein during the industrial acidulation of raw soapstock. In fact, the reaction between lutein and acids proceeds readily and we have achieved considerably higher yields than those reported (*4*), by refluxing lutein or lutein-containing materials, such as dehydrated alfalfa meal, with benzene and *p*-toluenesulfonic acid. This has enabled us to prepare sufficient amounts of the provitamin for feeding tests with chicks. We now have evidence that the chick converts the pigment to vitamin A_2.

For the preparation of the provitamin 30 g for a lipid extract from alfalfa (*7*), containing 3.87 mg β-carotene, 3.59 mg lutein and 0.38 mg zeaxanthin per gram, were refluxed for 30 minutes with 300 ml benzene and 2 g *p*-toluenesulfonic acid. Saponification was effected by adding to the mixture 300 ml of ethanol and 30 ml of KOH solution (50 g per 100 ml) and refluxing for 20 minutes. After cooling, water was added until separation of two phases occurred, the benzene phase was removed, and the aqueous alcohol phase was reextracted with benzene until the extracts were colorless. After washing and drying of the combined benzene solutions, and after removal of the

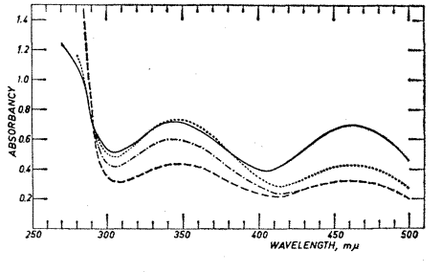

Fig. 1. Absorption spectra in ethanol of liver lipids from four vitamin A deficient chicks, after dosing with the following amounts of provitamin A_2: 5.4 mg (—·—· and — —), 8.4 mg (———), and 10.8 mg (– – – –).