The change in chromosomal complement of tissue culture cells occurs slowly or rapidly with age[6,7,11]. In Citrus fruit tissue, culture change from essentially diploid cells to predominantly polyploid cells was brought about within 6 months[7]. But in the present investigation this shift in higher polyploids and aneuploids was brought about within 24 months.

Zusammenfassung. In Kallusgewebekulturen von *Nigella sativa* nimmt mit der Zeit der Anteil der diploiden Zellen ab, während sich die Anteile von aneupliden, von 2*n* abweichenden Zellen vermehren.

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Influence of Nutritional Factors on Chick Epidermal Differentiation

Differentiation of chick epidermis in vitro can be influenced by environmental factors such as the nutrients. Indeed, 5–6-day chick embryo skin keratinizes in a natural nutritional medium (chick embryo extract and chicken plasma)[1,2]; it does not if maintained with a protein-free chemically defined medium[3], which is able to support differentiation of 12-day embryo skin[3]. This different behavior suggests the possibility that embryo extract and/or adult serum contain differentiation promoting factor(s), which is (are) no longer necessary at 12 incubation days.

We have therefore examined the ability of adult serum, 6-day and 12-day chick embryo extracts to support development in vitro of 6-day incubation skin and the relationship between keratinization and cell division. To make comparison with these processes in vivo, we have also studied skin histodifferentiation in situ. As a criterion of proliferation we took the number of mitosis in the epidermis; as the criteria of differentiation, the epidermis structure, the presence of birefringent material and the presence and distribution of sulphidrils (SH)[3].

Methods. For studying normal differentiation in vivo, fragments from the thigh regions of 6-, 8-, 10-, 12-day White Leghorn embryos were used. Explants were derived from the same regions of 6-day chick embryos. Skin areas were removed under sterile conditions, rinsed in Tyrode's, carefully dissected and then placed in culture dishes on the vitelline membrane, according to WOLFF[4]. We carried out 5 sets of experiments using the following nutritional media: 12-day embryo extract and chicken serum (thereafter E_{12}+CS), 6-day embryo extract and chicken serum (E_6+CS), 12-day embryo extract (E_{12}), 6-day embryo extract (E_6) and chicken serum (CS) (see Table). Chick embryo extracts were prepared in our laboratories as follows: eyes removed and discarded, embryos pressed out through stainless steel grid in sterilized cylinder, equal volume of Tyrode's added, centrifuged and supernatant used.

In order to ascertain the importance of the presence of adult serum in nutritional medium, some cultures, after 24 and 48 h incubation in E_{12}+CS or E_6+CS, have been transferred to E_{12} or E_6.

Cultures were incubated at 37 °C for more than 6 days. Every day, tissues were fixed in Bouin's fluid and routine histological procedures were followed. Serial sections (8–10 μm) stained with hematoxylin-eosin were used for morphological examinations and thicker sections (25 μm mounted in 10% NaCl solution) for studying birefringence by means of a Leitz polarizing microscope. SH groups were localized with the ferric ferricyanide method according to CHÈVREMONT and FREDERIC[5]. Mitotic counts were performed in epidermal layers along the basement membrane, using an ocular micrometer.

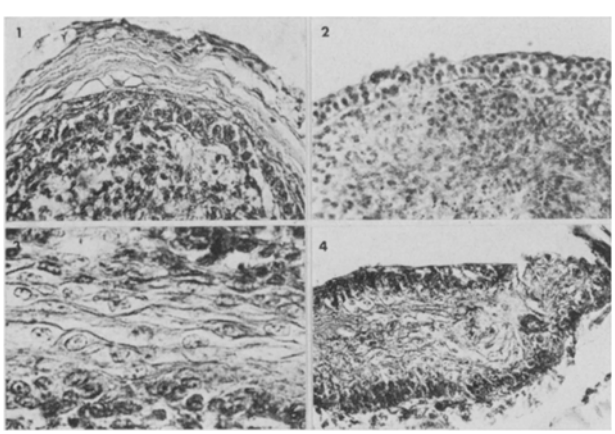

Fig. 1. 6-day skin explant after 6 days of incubation in E_{12} + AS. ×256.
Fig. 2. 6-day skin explant maintained in E_{12} for 6 day. ×125.
Fig. 3. 6-day skin culture after 4 days of incubation in CS; note the presence of a squamous layer. ×625.
Fig. 4. 6-day skin explant after 6 days in E_{12} nutritional medium to show the growth of the epidermis directly over the vitelline membrane (bottom) and the epidermal keratinization. ×125.

Composition of nutritional media

Media	Gelose 1% in Gey fluid	12-day embryo extract	6-day embryo extract	chicken serum (Difco)	Penicillin (10 Oxford U) in Tyrode's
E_{12} + CS	6[a]	3	–	3	1
E_6 + CS	6	–	3	3	1
E_{12}	9	3	–	–	1
E_6	9	–	3	–	1
CS	9	–	–	3	1

[a] Number of drops.

[1] B. MISZURSKI, Arch. exp. Zellforsch. *20*, 122 (1937).
[2] C. B. MCLOUGHLIN, J. Embryol. exp. Morph. *9*, 370 (1961).
[3] N. K. WESSELLS, Devel. Biol. *3*, 355 (1961).
[4] E. WOLFF, Devel. Biol. *3*, 767 (1961).
[5] A. G. E. PEARSE, *Theoretical and Applied Histochemistry* (J. Churchill, London 1968), vol. 1, p. 621.

Results. Epidermal differentiation in vivo. Our results on normal epidermal histodifferentiation closely agree with those of previous reports[2,3]. The covering epithelium of the 6-day-old chick embryo consists of 2 layers: a basal layer of columnar cells and a superficial layer of flattened cells. The basal layer rests on a basement membrane. The underlying mesenchyme is loose; mesenchymal cells are packed near the basement membrane. As development proceeds, mitotic rate increases so the three-layered condition is attained at 11–12 days (basal layer, intermediate layer, periderm, Figure 5). Birefringent material is detectable in the periderm at 12 days. SH groups reaction is weakly positive only in the periderm region.

Epidermal differentiation in vitro. E_{12}+CS. In the explants maintained in this nutritional medium, epidermis keratinizes within 5–6 days of incubation. After 48 h, the epidermis still consists of 2 layers. By the 3rd–4th day a squamous layer appears, periderm thickens and a subperidermal layer becomes present. By the 5th–6th day keratin makes its appearance in the subperidermal region; the periderm is still retained (Figure 1). A weak reaction for SH groups is observed in the periderm after 3 days of culture. As incubation proceeds, reaction is strongly positive and is detectable also in the squamous layer. Birifringence is clearly detectable in the periderm and subperiderm region after 6 days.

E_6+CS. Cultures supplemented with this nutrient exhibit a behaviour very similar to that described above. E_{12}. After 2 days of culture in E_{12} nutritional medium, skin explants show a two-layered epidermis. Mitoses are frequent (Figure 5). By 5–6 days the layers are 3 to 4, periderm is evident, the squamous layer is absent (Figure 2). Periderm exhibit a weak reactivity for SH groups and a birefringence. E_6. The findings are analogous to that described for E_{12} supplemented cultures.

CS. As the E_{12}+CS and E_6+CS maintained cultures, the explants supplemented with this medium undergo keratinization within 6 days. By the 3rd day, the epidermis consists of 3 layers; a squamous layer begins to appear after 4 days (Figure 3). Periderm and subperiderm show a positive reaction for SH groups after 3 days. By the 5th day subperiderm, being keratinized, strongly stains, while squamous layer is weakly reactive. By 5–6 days a birifringent material is detectable.

Skin explants maintained in E_{12}+CS (or E_6+CS) for 24 or 48 h and then transferred in E_{12} (or E_6) fail to keratinize. After 6 days, the epidermis is healthy and consists of 3 epithelial layers.

Finally we call attention to a characteristic behaviour of the epidermis in culture. After 2–3 days, epidermis overgrows more than the underlying mesenchyme so as to become sited directly over the vitelline membrane. In this condition, epidermis undergoes keratinization whatever nutritional medium is administrated (Figure 4).

Discussion. 6-day chick embryo skin if maintained in a suitable nutritional medium keratinizes in vitro faster than in vivo; it is therefore a self-differentiating system. Epidermis when grown directly over the vitelline membrane keratinizes regardless of the culture media employed. Epidermis in contact with mesenchyme develops in different ways according to nutrients; it undergoes differentiation if maintained with adult serum containing media, it does not in adult serum lacking media. These findings together with previous observations (ability of isolated[1] or combined with frozen-killed[6] epidermis to differentiate when cultured in natural nutrients and its inability if maintained in protein-free medium[7]) demonstrate that a) epidermis possesses the complete equipment for keratinization; b) macromolecular fractions, contained both in 6-, 12-day embryo extracts and chicken serum, are necessary to evoke epidermal differentiation[6,7]; c) mesenchyme controls epidermal differentiation; d) mesenchymal control is related to nutritional factors and then to cell activities. Moreover, the fact that in the same nutrients (serum lacking media) isolated epidermis (e.g., sited directly over the vitelline membrane) is able to keratinize, while epidermis resting over mesenchyme is unable, indicates that mesenchyme exerts an inhibitory effect, which is removed by a substance present in adult serum. This factor needs to be supplied for a certain time (cultures transferred after 48 h in medium lacking chicken serum fail to keratinize). Therefore, the previously observed ability of natural nutrients to support epidermal differentiation[1,2] is to be referred to adult serum factors(s).

As concerns the relationship between cell division and differentiation, these processes, at least in vitro, are not closely related; mitotic frequence exhibits the same pattern, whether epidermis keratinizes or not (Figure 5)[8].

On the basis of the present results and the available data[9–11], we suggest that mesenchyme acts on epithelium through 2 different mechanisms. First, mesenchyme stimulates epithelial mitotic activity; this action is unspecific and widespread as demonstrated by epidermis-heterotopic mesenchymes recombinations[11,12]. Second, mesenchyme specifically acts upon epithelial differentiation.

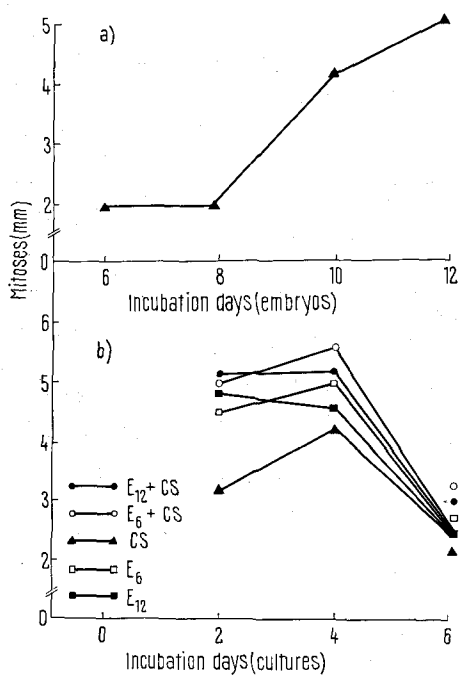

Fig. 5. Number of mitosis/mm: a) in the developing chick embryo epidermis; b) in the 6-day explanted epidermis for the indicated days of incubation.

[6] J. W. DODSON, J. Embryol. exp. Morph. *17*, 83 (1967).
[7] N. K. WESSELLS, Proc. natn. Acad. Sci., USA *52*, 242 (1964).
[8] N. K. WESSELLS, Expl. Cell Res. *30*, 36 (1963).
[9] S. ROTHBERG and T. M. EKEL, Nature, Lond. *216*, 1352 (1967).
[10] S. ROTHBERG and T. M. EKEL, Nature, Lond. 329, 341 (1971).
[11] C. B. MCLOUGHLIN, J. Embryol. exp. Morph. *9*, 385 (1961).
[12] N. K. WESSELLS, Devel. Biol. *4*, 87 (1962).

This activity is dependent upon cell metabolism and activated by environmental factors[13].

Riassunto. E'stata esaminata l'azione di estratti embrionali di 6 e 12 giorni e del siero di pollo sul differenziamento in vitro di epidermide di 6 giorni di incubazione. I risultati ottenuti hanno permesso di precisare alcuni aspetti del rapporto epitelio-mesenchima nel corso del differenziamento della cute.

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3 September 1971.

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Synaptonemal Complexes in the Ovaries of *Galleria mellonella*

The multiplication of the oogonial cells – cytoblasts – in insects ovaries (according to the terminology of KOCH et al.[1]) takes place during the course of the larval life. The nuclear events in the ovary which follow this period are: 1. polyploidization of the cells which become trophocytes and 2. onset of meiotic prophase of those which are becoming oocytes. In *Drosophila* these differentiation processes begin during the pupal stage and continue throughout the adult life[1]. In Lepidoptera, according to DEPDOLLA[2] the oogonial cell divisions last until the 4th larval stage. The number of larval stages, however, is not fixed for this group. It varies in different species depending on the environmental conditions. In *Galleria mellonella* the cytological observation of the ovary from spinning larvae taken from the cocoon indicate that the process of oocyte differentiation, namely meiosis, may start prior to pupation. In such ovaries, the nuclei with synaptonemal complexes may be found.

Material and methods. The larval ovaries were fixed in 2% glutaraldehyde, pH 7.3 buffered with Na-cacodylate, and postfixed, after washing, with 1% osmium tetroxide. Epon embedded material was sectioned on Reichert

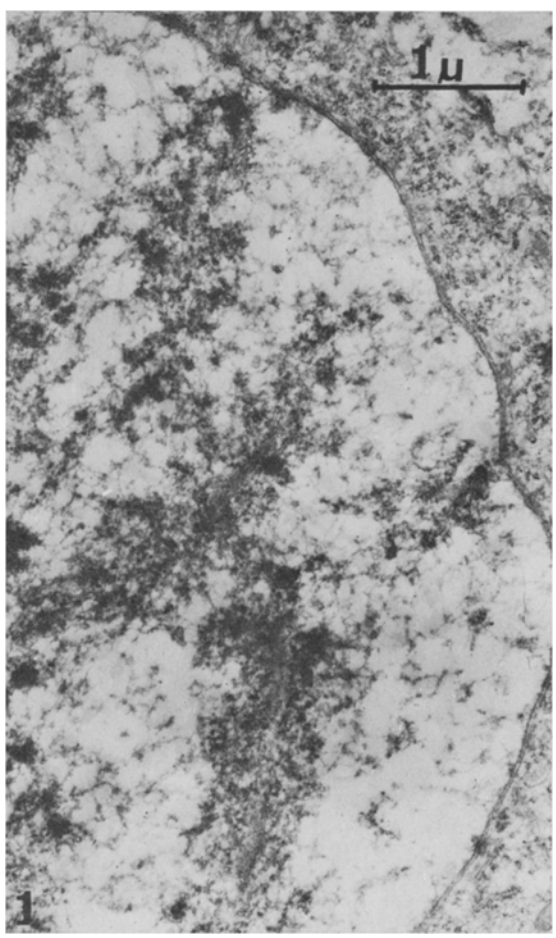

Fig. 1. Ovary of a spinning larva of *G. mellonella*. Fragment of an oocyte nucleus with synaptonemal complexes.

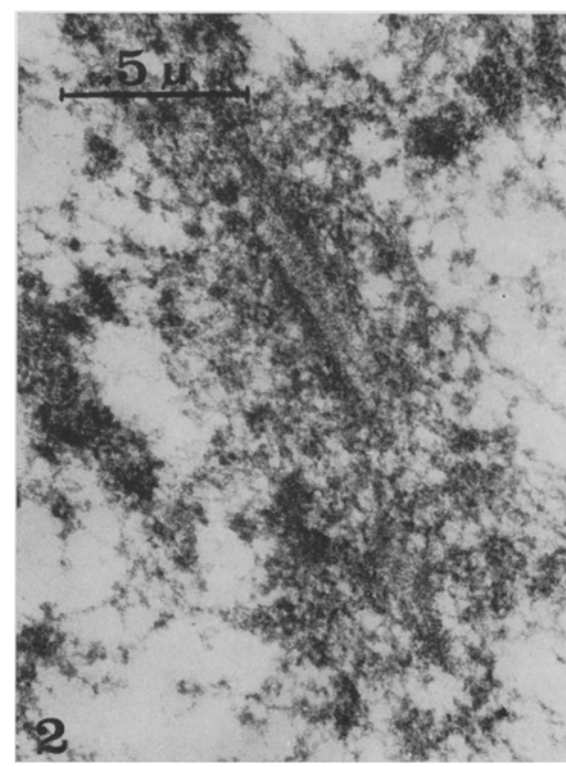

Fig. 2. Ovary of a spinning larva of *G. mellonella*. Another example of a synaptonemal complex in oocyte nucleus.

[1] E. A. KOCH, P. A. SMITH and R. C. KING, J. Morph. *121*, 55 (1967).
[2] Ph. DEPDOLLA, in *Handbuch der Entomologie* (Ed. Ch. SCHRÖDER, Jena 1928).