Study of yolk-sac endoderm organogenesis in the chick using a specific enzyme (cysteine lyase) as a marker of cell differentiation

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SUMMARY

The detection of a specific enzyme (cysteine lyase) of the yolk-sac endoderm by a very sensitive method is employed to characterize cell differentiation during the early stages of endoderm organogenesis in the chick.

The first cells to contain active cysteine lyase are found in the germ wall at the primitive streak stage.

In vivo observations establish a relation between the morphological specialization and organization of endodermal cells, their loss of mitotic activity and the increase in cysteine lyase activity. They suggest an influence of the mesoderm on endoderm differentiation.

In vitro experiments confirm the existence in the yolk-sac endoderm of an incompatibility between cell proliferation and differentiation, as well as the action of the mesoderm on both the structural organization of the endoblast and the appearance of cysteine lyase; this last action seems to be due mainly to blood cells; chicken and rabbit blood cells are equally active.

The problems of the origin of the endoderm and of the interactions occurring during the organogenesis of the yolk-sac endoderm are discussed.

INTRODUCTION

Little information is available on the mechanisms involved in the succession of processes occurring during the early stages of embryonic development which lead to the morphological and physiological specialization of cells and their organization into functional structures. Chick yolk-sac endoderm appears to be a particularly suitable material for approaching such problems, since endodermal cells are easily identifiable in the non-incubated germ, and different stages of endoblast differentiation coexist on a single embryo because of the centrifugal growth of the yolk sac; moreover, the development of the endoderm function can be followed by the presence of specific enzymes.

The existence of a specific enzymic system allowing the reduction of oxidized inorganic sulphur and its utilization for the synthesis of organic-sulphur-
containing compounds has been discovered in the chick yolk-sac (Chapeville & Fromageot, 1967). Although similar systems are widespread in micro-organisms and plants, they have never been found in an animal tissue except in the yolk-sac endoderm of birds and tortoises. The reactions leading from sulphate to cysteic acid and to taurine in the chick yolk-sac are summarized in Fig. 1.

The enzymes which catalyse reactions 4 and 5 are also present in the liver of the embryo as well as of the adult chicken (where taurine synthesis proceeds by progressive oxidation of the cysteine sulphur without rupture of the C—S bond), whereas the enzymes which catalyse reactions 2, 3 and 6 are specific to yolk-sac endodermal cells. Cysteine lyase, which catalyses the substitution of the cysteine thiol group by organic sulphite to produce cysteic acid (Fig. 2), is synthesized in large amounts and can be detected histochemically by a very sensitive method, consisting of trapping in situ the liberated hydrogen sulphide with lead acetate to form a dark precipitate of lead sulphide. It should be pointed out that, although inherently of great interest on account of its vestigial character, cysteine lyase is only used here as a tool to characterize cell differentiation.

We shall report experiments relating to the localization of the first cells to contain cysteine lyase, and to the study of in vivo and in vitro differentiation of the yolk-sac endoderm; in this last part, the influence of mesoderm (particularly of blood cells) is examined.
MATERIALS AND METHODS

Eggs of White Leghorn chickens were used for the experiments. Tissue fragments were cultivated on embryo extract-gelose medium (Wolff & Haffen, 1952).

In histochemical studies, embryos or explants were fixed in cold 90% ethanol for 30 min to 1 h, and embedded in paraffin after rapid dehydration, using benzene as the paraffin solvent. Cysteine lyase activity is visualized on slides incubated at 37 °C in a reaction mixture containing L-cysteine $10^{-2}$M, sodium sulphite $10^{-2}$M, lead acetate $10^{-3}$M and pyridoxal 5-phosphate $10^{-5}$M in a Tris-HCl solution (0.15M, pH 8.5). Products were purchased from Merck. This method is sensitive enough to detect a few active molecules of cysteine lyase. A long incubation is used for the detection of low enzyme activity whereas a short incubation reveals differences between various regions of an embryo or explant, or between various explants in which the enzyme activity is high.

In autoradiographic studies, samples were fixed in cold 95% ethanol-acetic acid (3:1), after being incubated for 1 h on gelose medium containing 30 μCi of [Me-3H]thymidine (spec. act. 6.3 or 9.3 Ci/mM; Section des molécules marquées, Saclay, France) per ml of medium. Slides were submitted to a 5% perchloric acid hydrolysis before dipping in K 5 Ilford Gelform Emulsion (Caro & Van Tubergen, 1962).

Chick embryo blood was taken from yolk-sac veins at less than 10 days of incubation with a capillary pipette. Adult chicken blood was obtained by cardiac puncture with 1% of trisodium citrate. Plasma was collected after centrifugation at 1000 g for 10 min and cells were rinsed in Tyrode's solution.

RESULTS

1. Localization of the first cells to contain active cysteine lyase

The presence of cysteine lyase was investigated in chick embryos from the non-incubated germ. The first stage at which the enzyme was detected is the advanced primitive streak stage, where cysteine lyase appears in a few cells dispersed in the germ wall (Fig. 3). From this stage the enzyme activity increases in the central region of the area opaca, while enzyme-containing cells are detected in more and more distal regions, although staying approximately in the interior half of the blastoderm. Prior to the establishment of blood islands, cysteine-lyase-containing cells do not differ in appearance from other cells, with the exception of the cells of the peripheral region (cf. §2.1).

The experiments indicating that the appearance and increase of cysteine lyase activity are due to the biosynthesis of the enzyme and not to the activation of a pre-existing protein will be reported elsewhere. They have shown that, when extracts from yolk-sac cells in which cysteine lyase is absent are incubated with extracts from cells producing cysteine lyase, there is no increase in cysteine lyase activity.
2. In vivo differentiation of the yolk-sac endoderm

2.1. Morphological aspect. After blood island formation, the endoderm can be divided into four concentric regions corresponding to distinct steps in cellular differentiation and organization (Figs. 4, 5). In the inner zone (zone 1) yolk drops have been transformed into substances which are extracted during fixation and embedding, and cells have become arranged into a single-layered epithelium presenting a morphological polarity: hypertrophied nuclei lie near the base of the cells (i.e. on the side of the vascular system), where a basement membrane and desmosomes have been described (Bellairs, 1963). By the time the embryo has about ten somites, the epithelium has reached the edge of the area vasculosa, with which it will coincide from this stage. Zones 2 and 3 (area vitellina) are composed of large cells swollen with yolk drops and irregularly packed in several layers. Zone 4 consists of a single layer of small cells with small nucleus and poorly developed cytoplasm apparently deprived of yolk drops. We consider
Chick yolk-sac endoderm differentiation

these cells to be endodermal since they have been shown to contain cysteine lyase after a certain period of culture. Before blood island formation, only the last three zones are present.

2.2. Cell proliferation. Embryos having about ten somites were incubated for 1 h in the presence of [³H]thymidine. Nuclear counts on autoradiographs obtained from these embryos show that the ratio of labelled nuclei, and hence the mitotic activity, increases from the pellucida–opaca junction to the edge of the blastoderm. Under the experimental conditions described, [³H]thymidine was incorporated by 4% of the cells in zone 1, 30% in zone 2, 43% in zone 3 and 76% in zone 4 (Fig. 6).

2.3. Appearance of cysteine lyase. At the ten-somite stage, cysteine lyase activity is very high in the area vasculosa (zone 1), and drops considerably on the other side of the sinus terminalis (zone 2) (Fig. 7). No cysteine lyase is detected in zones 3 and 4, either on slides or on non-fixed embryos. If slides are incubated for a short time in the reaction mixture, the enzyme is first detected at the base of the cells of the epithelium (i.e. on the side of the vascular system). It can be noted that, at the primitive-streak stage, cysteine lyase first appears in cells lying near the mesodermal layer, which has just begun to invade the posterior part of the area opaca (Fig. 3C).

The centripetal differentiation of the yolk-sac endoderm in vivo can thus be described by three apparently correlated events: the progressive loss of the mitotic activity, the morphological differentiation of endodermal cells and their organization into an epithelium, and the increase of cysteine lyase activity. The ‘differentiated state’ (structural changes, cessation of DNA synthesis and high cysteine lyase activity) is reached only when the endoderm is colonized by the mesoderm (zone 1); this suggests the existence of an interaction between the two tissues.

Figures 5–7

Fig. 5. Different steps in endoderm morphological differentiation in a 2½-day-old embryo (fix. Bouin).
(A) Area vasculosa: cells are arranged in a single-layered epithelium. Hypertrophied nuclei lie at the base of the cells; most of the cell cytoplasm is occupied by large vacuoles. x 1050.
(B) Area vitellina: yolk-loaded cells are irregularly packed in several layers. x 1125.
(C) Peripheral zone: small cells with small nuclei are arranged in a single layer.

Fig. 6. Autoradiographs of a ten-somite embryo incubated for 1 h in the presence of [³H]thymidine (30 μCi/ml).
(A) Area vasculosa: less than 5% nuclei were labelled. x 450.
(B) Area vitellina: 30% nuclei were labelled. x 450.

Fig. 7. Detection of cysteine lyase at the area vasculosa/area vitellina junction. The enzyme activity is very high in the area vasculosa, and considerably lower on the other side of the sinus terminalis (st). The embryo had ten pairs of somites. a.va = area vasculosa; a.vi = area vitellina. x 330.
3. In vitro differentiation of yolk-sac endoderm

3.1. Cell proliferation and cysteine lyase activity. The ability of the area vitellina interna (zone 2) to differentiate in vitro was investigated. In this region, according to §2, endodermal cells are not organized, 30% of them synthesize DNA, and cysteine lyase begins to be detectable.

Fragments of zone 2 of embryos having about ten somites were cultivated. After various culture durations, proliferation and cysteine lyase activity were examined in the following manner: one fragment was transferred onto a $[^3H]$thymidine-containing medium in order to obtain autoradiographs, while another was treated for the detection of cysteine lyase. Results are shown in

\[ \text{Figure 9} \]

Increase of cysteine lyase activity in endodermal cells as a function of culture duration. Fragments of the area vitellina interna (zone 2) of a ten-somite embryo were cultivated for 0 h (A), 7 h (B) and 24 h (C). Slides were incubated for 6 h in the reaction mixture. Cysteine lyase activity is visualized by dark spots of lead sulphide. Estimation of $[^3H]$thymidine incorporation in corresponding fragments of the same embryo (cf. Fig. 8) show that the increase in cysteine lyase activity is concurrent with the decrease of DNA synthesis. $\times 340.$
Fig. 10. Schematic representation of in vitro association of fragments of the area vitellina interna with the vascular system of the same embryo. Fragments of the area vitellina interna are associated with the vascular system so that the face of the endoderm normally touching the yolk touches the mesoderm. $a.en =$ Associated endoderm; $o.en =$ original endoderm; $m =$ mesoderm; $ec =$ ectoderm.

Figs. 8 and 9. Whereas DNA synthesis decreases as a function of culture duration, the amount of cysteine lyase increases. The presence of important amounts of a specific protein being usually considered as a criterion of cell differentiation, these results confirm the existence in the yolk-sac endoderm of an incompatibility between cell proliferation and differentiation, and can be related to many observations made on various tissues (Cahn, 1968). Although a highly quantitative study of the cysteine lyase activity has not been attempted, it is clear that it is considerably increased after 24 h of culture, when almost no $[^3]$H]thymidine is incorporated, just as it occurs in vivo in the endoderm of the area vasculosa. It can thus be assumed that the evolution of the physiological activity of endodermal cells from the area vitellina in vitro is similar to the in vivo evolution. However, after 24 h of culture, endodermal cells have not arranged themselves into an epithelium. This fact as well as the numerous observations made in vivo (cf. §2) strongly suggests that the mesoderm plays a morphogenetic role in the endoderm differentiation.

3.2. Tissue organization and cysteine lyase activity. The influence of the mesoderm was tested in vitro using 3-day-old embryos, by opposing fragments of the area vitellina interna (zone 2) to the vascular system of the same embryo so that the face of the endoderm normally touching the yolk touches the mesoderm (Fig. 10). After 5 h of culture both tissues strongly adhere to each other, and cysteine lyase activity is higher in endodermal cells lying close to blood vessels (Fig. 11A). After 48 h of culture the associated endoderm has become
Chick yolk-sac endoderm differentiation

Table 1. Action of embryo and adult chicken blood on the appearance of cysteine lyase

Fragments from the peripheral zone of 16 embryos having about 10 somites were cultivated for about 20 h either alone or with blood cells or plasma. Cysteine lyase activity in these fragments is indicated as follows: − if no precipitate of lead sulphide is visible; (+) if it is present in a few cells; +, if it is light but present in many cells; ++, if it is very important.

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<th>Cysteine lyase activity</th>
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a single-layered epithelium with basal nuclei, in which many yolk drops have been partly digested (Fig. 11B). In the presence of mesoderm therefore, the endoderm of the area vitellina interna can reach its fully differentiated state in vitro. Moreover, the mesoderm is capable of inverting the morphological and physiological polarity of the tissue.

The question arises as to which constituent of the vascular system is responsible for this action. In some places, blood-vessel endothelium is seen to project into the endodermal layer as if starting to form folds or villi, similar to those observed in vivo after 3 or 4 days of incubation. The endothelium might therefore be responsible for the formation of endoderm folds; it might also help in the formation of the epithelium by stretching the endodermal layer. Further investigations were made concerning the activity of blood constituents.

3.3. Action of blood on the appearance of cysteine lyase. Fragments of the peripheral zone of ten-somite embryos were cultivated in the presence of embryo or adult chicken blood. Blood cells tend to gather in islets which sink in the endodermal layer. Cysteine lyase begins to be detectable in endodermal cells situated close to these islets after 5 h of culture, and its amount increases rapidly during the following hours (Fig. 12).

In another experiment, peripheral fragments of the same embryo (about ten somites) were cultivated on embryo extract/gelose medium, either alone or with blood cells or plasma, for about 20 h. The results are summarized in Table 1. They were similar when, instead of chick embryo, adult chicken blood was used. In both cases the appearance and the increase of cysteine lyase activity are rapid in the presence of blood cells and much slower in the presence of plasma. Contact between endodermal and blood cells does not seem necessary, as the same effect is obtained when blood cells are included in the gelose medium; and as cysteine lyase activity is increased in endodermal cells separated from blood cells by the ectodermal layer. A diffusible factor seems therefore to be responsible for this
action. The slight action of blood plasma could be explained by the presence of a few cells, or that of a certain amount of an active factor liberated from the cells. It must be noted, however, that, even though much more slowly, cysteine lyase does appear in peripheral fragments cultivated alone; after 20 h the amount of enzyme is very low as compared with that observed in the presence of blood cells, but it can reach high levels after 2 or 3 days. The embryo extract (which might contain a possible active mesodermal factor) present in the medium is not responsible for the appearance of cysteine lyase in such fragments, since the enzyme also appears in fragments cultivated without embryo extract.

No organization into an epithelium was observed in these experiments.

In a few experiments, endoderm of the area vitellina interna or of the peripheral zone was associated with other types of cells. Ectoderm, somites, or fragments of limb-buds (the last two from mesodermal origin) seemed to have no effect on endodermal cells. However, rabbit blood cells acted as chick blood cells on the increase of cysteine lyase, indicating that erythrocyte nuclei are not directly involved.

Further experiments should allow the identification of the factor present in blood cells, and the determination of its level of action, but it should be kept in mind that the occurrence of regulative processes in tissue fragments separated from the blastoderm cannot be excluded.

**DISCUSSION**

1. *Origin of the endoderm*

Since cysteine lyase is a specific enzyme of the yolk-sac endoderm, the fact that the cells in which it is first detected are situated in the germ wall is consistent with the generally accepted concept of the germ-wall origin of the extra-embryonic endoblast, a concept which implies a centrifugal migration of the cells. Our results are also compatible with the concept of an origin from the periphery.

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**Figures 11 and 12**

Fig. 11. Association of a fragment of inverted area vitellina with the vascular system of the same embryo (cf. Fig. 10).

(A¹) After 5 h of culture cysteine lyase activity is higher in endodermal cells lying close to blood vessels. × 112.

(A²) The same; higher magnification. × 1125.

(B) After 48 h of culture the associated endoderm has become arranged into a single-layered epithelium with basal nuclei; yolk drops have been partly digested. × 1125.

The embryos were 3 days old. *a.en* = associated endoderm; *ec* = ectoderm; *bv* = blood vessel; *a.ec* = associated ectoderm; *en* = endoderm.

Fig. 12. Culture of a fragment of the peripheral zone of a ten-somite embryo in the presence of embryo blood. Cysteine lyase activity is very high in endodermal cells surrounding the blood cell islets (*bc*), but not detected in other parts of the explant. The culture duration was 20 h. × 240.
of the blastoderm by surface material rolling round the margins and centripetal migration of the endoblastic cells (Lutz, 1955). In both cases the rapidly proliferating cells are expected to be found in the external part of the area opaca, and the more differentiated cells in its internal part.

However, since no cysteine lyase is detected in the embryonic endoderm, our results appear not to be in agreement with Lutz’ conclusion of a direct continuity between embryonic and extra-embryonic endoderm, and would rather support the hypothesis that these formations are of different embryonic origin. While Modak (1966) found that in hypoblast-deprived embryos the regenerating layer is formed both from cells invaginated through the primitive streak and from cells derived from the inner germ wall, most experiments carried out under normal conditions effectively suggest that the embryonic endoblast originates from the base of the primitive streak by gastrulation (Hunt, 1937; Spratt & Haas, 1965; Rosenquist, 1966; Vakaet, 1970; Nicolet, 1970). It should also be noted that no cysteine lyase has been detected in primordial germ cells located either in the endophyll or in the extra-embryonic endoderm.

2. Interactions occurring during the organogenesis of the yolk-sac endoderm

Several authors (Bremer, 1960; Mato, Aikawa & Kishi, 1964) have suggested the existence of an interaction between the endoderm and the mesoderm in the yolk sac. Our in vivo and in vitro observations show that the mesoderm exerts an influence on both the differentiation of the endodermal tissue (organization of the cells into an epithelium) and the differentiation of the cells themselves (increase in cysteine lyase activity). While the appearance of cysteine lyase is observed in endodermal cells cultivated alone, and amplified or accelerated in the presence of the vascular system or of blood cells, the arrangement into an epithelium was only obtained in the presence of the mesoderm. This might indicate that the processes leading to these two changes are distinct. However, since in vivo the formation of the epithelium follows the appearance of cysteine lyase, it can be argued that the cultures in the presence of blood cells were not carried far enough to allow the organization of the tissue; morphogenetic movements might also be inhibited under the culture conditions.

Yolk-sac endoderm has been reported to have a stimulating, but not essential, effect on the differentiation of blood cells (Miura & Wilt, 1969). Similarly blood-cell action on the endoderm appears as a stimulus which enables the cells to achieve their latent potentialities. Before colonization by mesoderm, intensive digestion of yolk by the endoderm would be useless as the products could not be carried to the embryo; on the contrary, as soon as blood islands are formed, and especially after the establishment of blood circulation, the breakdown of yolk becomes essential to nourish the cells of the embryo, which have by this time used up their own reserves (Bellairs, 1963). It could be suggested that the formation of the epithelium, which coincides with the occurrence of important structural changes in intracellular yolk, might be a consequence of the evolution
of the physiological activity of the cells induced by the blood cell stimulus. For example, the formation of the epithelium could be seen as a way of increasing yolk-sac efficiency (as later the formation of folds) that would occur when the enzymic machinery (of which cysteine lyase could be considered as an indicator) is functioning above a certain level. One observation can be interpreted as an argument in favour of this hypothesis: in vivo, before the mesoderm has invaded the anterior part of the area opaca (the proamnios), endodermal cells lining the cavity which exists between the endoderm and the ectoderm in this region are arranged into a polarized epithelium, and cysteine lyase activity is as high as in the area vasculosa. A factor liberated from blood cells could possibly diffuse from the area vasculosa into this cavity in sufficient quantity to produce the stimulus responsible for the increase in cysteine lyase activity. The fact that the endoderm of this region is organized in an epithelium suggests that there is a correlation between the increase of cysteine lyase activity and the morphogenetic role of the mesoderm.

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REFERENCES


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