THE DISTRIBUTION OF ASCORBIC ACID (VITAMIN C) IN THE EARLY STAGES OF THE DEVELOPING CHICK EMBRYO

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1. INTRODUCTION

In 1928 Szent-Györgyi published the results of researches on a strongly reducing substance in the cortex of the mammalian adrenal. This substance, which was then called hexuronic acid, was found to have reducing properties which exceed those of other known intracellular reducing agents: in particular, it is capable of reducing silver nitrate in the dark with great rapidity. Later, Harris & Ray (1933*b*), among other authors, showed that the substance is identical with the antiscorbutic vitamin C; the vitamin was therefore renamed ascorbic acid. On the basis of these studies a method has been elaborated, mainly by Bourne (1933*a*, *b*, *c*), Leblond (1934) and Giroud (1938), by which ascorbic acid can be specifically identified in histological preparations. The method has been applied to extensive investigations of the adult tissues of various species (see Giroud, 1938), but little use has hitherto been made of it in the study of embryonic cells and tissues. A survey has therefore been made of the distribution of ascorbic acid in developing chick embryos. The findings of this survey, in embryos up to the fourth day of incubation, are reported below.

2. METHODS

Silver nitrate is used by histologists in several ways. The method of Cajal for the staining of nervous tissue involves the fixation of the salt by the tissues, and its later reduction by a further reagent such as hydroquinone; this is also the case in Da Fano's method for Golgi substance. By contrast, the use of silver nitrate for the demonstration of ascorbic acid depends on direct reduction: in this case the *p*H of the silver nitrate solution becomes of primary importance. Silver nitrate in ammoniacal solution is very easily reduced, in neutral solution much less easily, whereas in acid solution it is reduced with comparative difficulty: thus according to Mellor (1923) it undergoes no reduction in solution with organic acids such as malic, tartaric, citric and malonic, even when boiled, unless there is an oxidising agent such as potassium permanganate present. There are inorganic substances, such as hypophosphorous and phosphorous acids, which reduce silver nitrate with the production of a black precipitate, but they are not of a kind which could exist free in living cells.

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In considering organic reducing agents it is important that the acid silver nitrate method for the demonstration of ascorbic acid involves the use of the reagent in definite physical conditions: it is used only in the cold, in the dark, and over short periods of time. In these conditions it is known that other strongly reducing substances which occur in cells have no effect on the reagent. Leblond (1934) has shown this to be the case for adrenalin, polyphenols such as tannin, various sugars and cysteine. Bourne (1936) removed the lipoids from cells, by means of chloroform, before subjecting them to the reagent, and found that the reaction persisted. On the other hand, washing with distilled water or any aqueous solution results in the disappearance of the reaction, owing to the removal of the vitamin in solution. Leblond (1934) also used methyl alcohol, the best solvent for ascorbic acid, in order to demonstrate the same effect: washing small pieces of tissue in methyl alcohol for 10 min. results in an alteration of the reaction; the reducing substance can be seen to have diffused through the tissue as a result of the mobilizing action of the solvent. Exposure to the solvent for longer periods causes the complete disappearance of the reaction.

There are two certain methods for the estimation of the amount of ascorbic acid present in a tissue: the first of these is investigation of its antiscorbutic activity, and necessarily does not give accurate results; the second is titration with dichlorophenolindophenol (Harris & Ray, 1933*a*). A number of studies has been made showing that histological findings regarding the presence of ascorbic acid parallel the estimates made by these methods. Leblond (1934) gives a table of both plant and animal tissues, including adrenal, corpus luteum, muscle, orange, cabbage and mushroom, in which this parallelism is illustrated. Further examples may be found in the work of Giroud (1938).

None of the above facts is conclusive. The most important evidence for the specificity of the silver nitrate reaction is derived from the studies of scorbutic animals (Leblond, 1934; Bourne, 1935*a*; Demole *et al.* 1935; Giroud & Leblond, 1936, 1937). Outside the primates only cavies are known to be susceptible to scurvy (i.e. to be unable to synthesize vitamin C), and the work has been done on them. In a variety of tissues, including the adrenal, the testis and the kidney, it has been found that the reaction with silver nitrate disappears progressively when the animal is put on a scorbutic diet. The results of an extensive study of the kidney were published by Giroud & Leblond in 1937. The reaction with silver nitrate was found to persist in the kidneys of rats on a scorbutic diet, but to disappear progressively from those of cavies on the same diet. Intravenous injection of 50 mg. ascorbic acid into scorbutic cavies resulted in the immediate reappearance of the reaction. Similar observations on a number of tissues had already (1936) been briefly reported on by these authors; they had also observed that injection of ascorbic acid into normal cavies caused the appearance of reactions in tissues, such as the liver and the epithelial cells of the intestine, which did not before show them.

It is known that certain melanin pigments constitute an exception to the

rule that only ascorbic acid reduces acid silver nitrate (Giroud & Leblond, 1936; Giroud, 1938, p. 22). The melanin granules in the Langerhans cells and the Malpighian layer of the skin show a reaction which persists both after extraction with methyl alcohol and during scurvy. These pigment granules are easily recognizable, and the reaction they give does not constitute an objection to the use of the reagent for demonstrating the presence of ascorbic acid in other tissues.

A further difficulty arises from the fact that the liver and adrenal medulla, both of which are known from biological and chemical tests to contain large quantities of ascorbic acid, show the reaction with silver nitrate only slightly or not at all (Bourne, 1933*c*; Galvao & Cardoso, 1934). Harris & Ray (1933*a*) have suggested that this is due to the presence of a substance which inhibits the oxidation of ascorbic acid. It is known that glutathione has a stabilizing action on ascorbic acid in vitro and in vivo (Borsook *et al.* 1937), and the question arose whether it might not be glutathione, or at least the presence of free sulphydryl, which was responsible for the supposed inhibition. Various authors (Emmerie, 1934; Svirbely, 1935; Giroud *et al.* 1936) have carried out experiments in vitro which show that glutathione can prevent the reduction of acid silver nitrate, in the relevant physical conditions, by ascorbic acid. The last-named authors report that the inhibitory effect becomes important when the concentration of glutathione reaches a level corresponding to one molecule to five of ascorbic acid. Giroud (1938, p. 34) concludes: "Ceci permet d'une part de supposer que des phenomènes d'inhibition puissent fréquemment jouer quand on réfléchit à la teneur relativement élevée en glutathione en divers tissus et d'autre part, oblige à envisager dans bien des cas processus d'activation pour expliquer des résultats positifs." This conclusion takes no account of the possibility that glutathione, even if it is the only inhibiting substance, may be present in some tissues in a state in which it is unable to exert its stabilizing action; it does, however, make clear that the mechanism underlying the failure of the reaction in liver and adrenal medulla is still far from fully established.

It remains to be pointed out that silver nitrate is only reduced by ascorbic acid itself,[1] and not by the vitamin in its reversibly oxidized form, dehydroascorbic acid. In all normal tissues which have been studied the latter occurs in a concentration only one-tenth to one-twentieth of that of the reduced vitamin (Giroud, 1938, p. 4). Bourne (1936) found that treatment of tissues with H_2S, which reduces dehydroascorbic acid before impregnation, has very little effect on the amount of silver precipitated.

As a result of these observations it has been generally concluded that the production of a black precipitate in tissues by the acid silver nitrate method indicates the presence of ascorbic acid (with the one exception which has been mentioned). The alternative possibility is that there occur, in some cells, reducing conditions which simulate the action of ascorbic acid on the reagent.

[1] The sodium salt of ascorbic acid (Roche), as prepared for injection, also gives a black precipitate with acid silver nitrate.

Although there is no evidence for this possibility, it cannot be wholly excluded for material to which the scurvy test cannot be applied. Nevertheless, for the following account it has been held justifiable to assume that the reactions observed are unlikely to be due to reducing substances other than ascorbic acid.

Details of method

The solubility of ascorbic acid makes it necessary to fix the tissues and to impregnate them with silver at the same time. Two mixtures were used for this purpose: the first was a 10 % solution of silver nitrate in 10 % acetic acid; the second was a saturated solution of silver nitrate in ethyl alcohol (5 parts), water (4 parts) and glacial acetic acid (1 part); the latter gives a concentration of silver nitrate of slightly less than 10 %. Both mixtures give good results, but with the second there is better fixation and rather better penetration. Lower concentrations of silver nitrate were tried, but were not found to be satisfactory. Embryos up to 24 hr. require only 15–20 min. in the mixture; larger embryos were given progressively longer, and at 4 days they were left in the fixative for up to 45 min. Fixation was carried out in the dark, after the embryo had been removed from the yolk and separated from its membranes. It was followed by thorough washing in distilled water, before dehydration and embedding in paraffin. All sections were cut at 5 μ, brought down to water and toned in very dilute gold chloride for from 4 to 10 min.; they were then left in sodium thiosulphate solution for a similar period, dehydrated, "cleared" in xylene and mounted in Canada balsam. The toning is not essential, but it removes the yellow-brown coloration of the tissues which tends to obscure the black granular precipitate of metallic silver.

Table 1

Unincubated	5
12 hr.	2
18 hr.	3
24 hr.	5
36 hr.	1
48 hr.	2
72 hr.	4
96 hr.	13

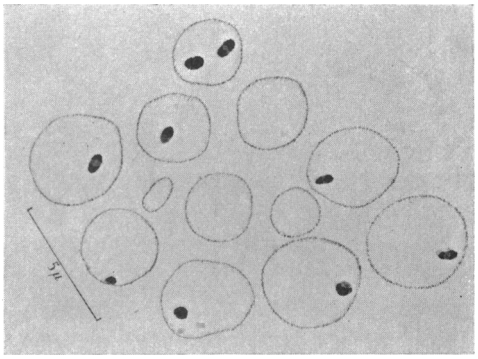
Table 1 shows the number of embryos used, with their ages. Embryos up to 24 hr. were first fixed *in situ* for 1 min. after removal of the albumen. This was done by holding over the yolk a piece of paper from which a small triangular section had been cut. A few drops of the fixative were allowed to come in contact with the embryo, which was in the uncovered, triangular area of the blastoderm. The fixed area was then cut out, with some yolk adhering to it, and left in the fixative for a further period of about 15 min. The shape of the triangle made possible the orientation of the embryo when it was embedded. Orientation in the first place was based on the relation of the blastoderm to

the poles of the egg. Embryos of from 1½ to 4 days were removed from the yolk with the whole blastoderm. The latter was floated out on distilled water, and held stretched by a small glass ring during fixation.

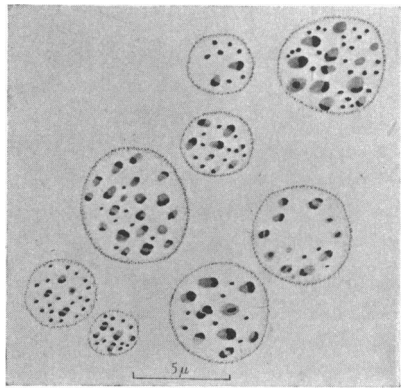
3. RESULTS

A. *Blastoderms before the appearance of an embryo*

Carrick & Hauge (1925) found that vitamin C is not present in the new-laid eggs of fowls kept on a scorbutic diet, but that its presence could be demonstrated in 5-day chicks hatched from such eggs. Ray (1934) was unable to find ascorbic acid in unincubated fowls' eggs by titration: titrable amounts appeared at the end of the fourth day of incubation, in the embryo but not in the yolk



Text-fig. 1. Extracellular yolk globules from unincubated egg. Small black deposits situated on a refractile granule (shown grey) represent ascorbic acid.

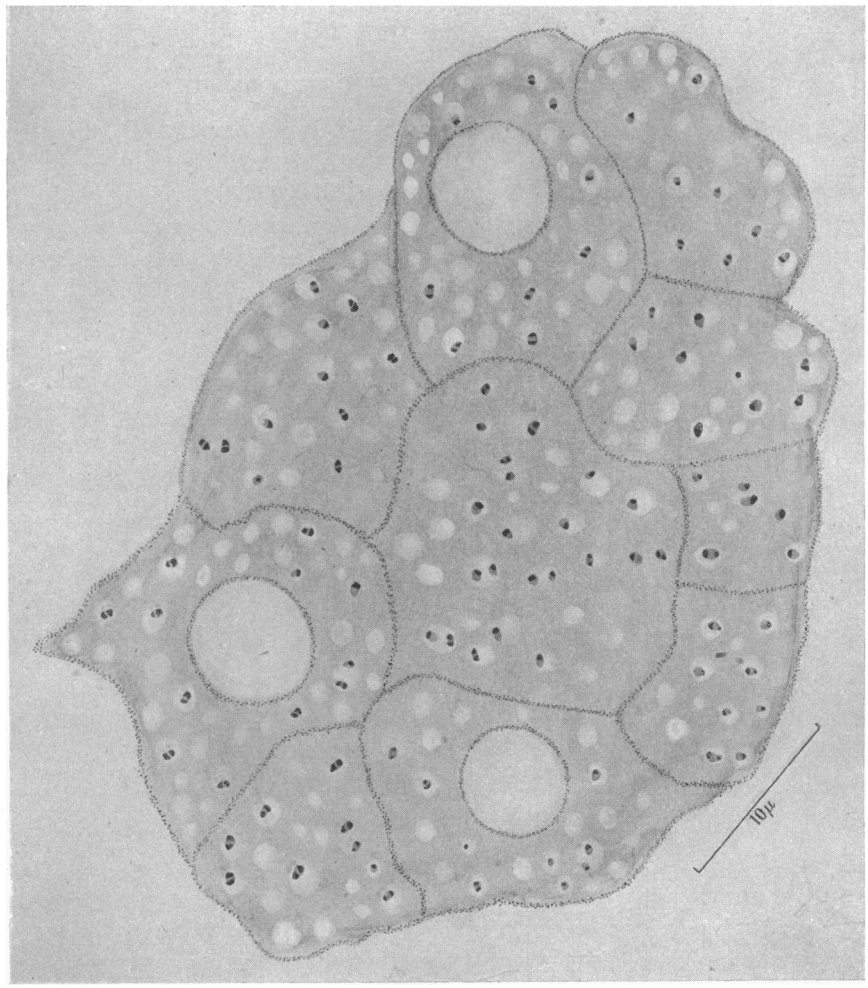


Text-fig. 2. Extracellular yolk globules from egg incubated for 18 hr. Note the greater number of granules.

or white. There is therefore no doubt that chick embryos early commence the synthesis of ascorbic acid. By using the acid silver nitrate method it has been possible to show that ascorbic acid has already appeared in the unincubated blastoderm, where it is present in granules of varying size attached to the surface of yolk granules. The latter may be within the cells of the blastoderm or in the layer of yolk which immediately underlies them; they may have several blackened granules attached to them, but generally at this stage they have only one (Text-figs. 1, 3, 8). It seems possible that the fact that ascorbic acid appears to be synthesized outside the blastoderm cells may be due to the diffusion of an enzyme from them into the yolk below.

By no means all the yolk granules have ascorbic acid in association with them. In the upper layer of the blastoderm (the epiblast) the black precipitate is confined to the periphery, and the remaining cells (which contain yolk) have no ascorbic acid. In the endoderm the diameter of the empty region is

smaller; in the layer of yolk granules below, the ascorbic acid may be evenly distributed, or it may be absent from a small central area (Text-fig. 8). It is clear that the region which is to form the area pellucida, and which will contain



Text-fig. 3. Endoderm cells from unincubated embryo. Small white globules represent intracellular yolk.

the primitive streak and the embryo, is in a different physiological state, with respect to ascorbic acid, from the rest of the blastoderm.

The small granules, attached to yolk globules, on which the silver is precipitated, frequently show irregular impregnations: often there are two black caps, crescentic in shape, at opposite poles of a granule, but there are many

variations (Text-figs. 1–3). It is not to be supposed that the exact form of the deposit is of great significance, since the precise arrangement of the molecules of precipitated silver will doubtless depend to a large extent on the physical conditions which prevail in the neighbourhood of the reducing agent.

At the 12th hour of incubation, i.e. at the stage at which the primitive groove appears, the distribution of ascorbic acid remains the same. At 18 hr., when the primitive streak reaches its greatest length, the black granules are confined to the yolk underlying the blastoderm. At this stage there is consequently no intracellular ascorbic acid; on the other hand the amounts associated with the yolk granules appear to be much greater (Text-fig. 2) than those in earlier blastoderms.

B. *Blastoderms after an embryo has developed*

Black deposits have reappeared in the cells by 24 hr. They are confined almost entirely to extra-embryonic tissues, and in particular to the ectoderm and endoderm. Owing to the unavoidably bad fixation obtained with the silver nitrate reagent, relatively few of the ectodermal and endodermal cells can be fully studied. Nevertheless, in the less distorted cells it can be clearly seen that the ascorbic acid is distributed in granules, spherical or rod-shaped, which occupy all regions of the cytoplasm: the cytological picture consequently resembles a mitochondrial impregnation (cf. Giroud, 1938, p. 26; Bourne, 1935*b*), the nucleus appearing as a clear space (Text-fig. 4).

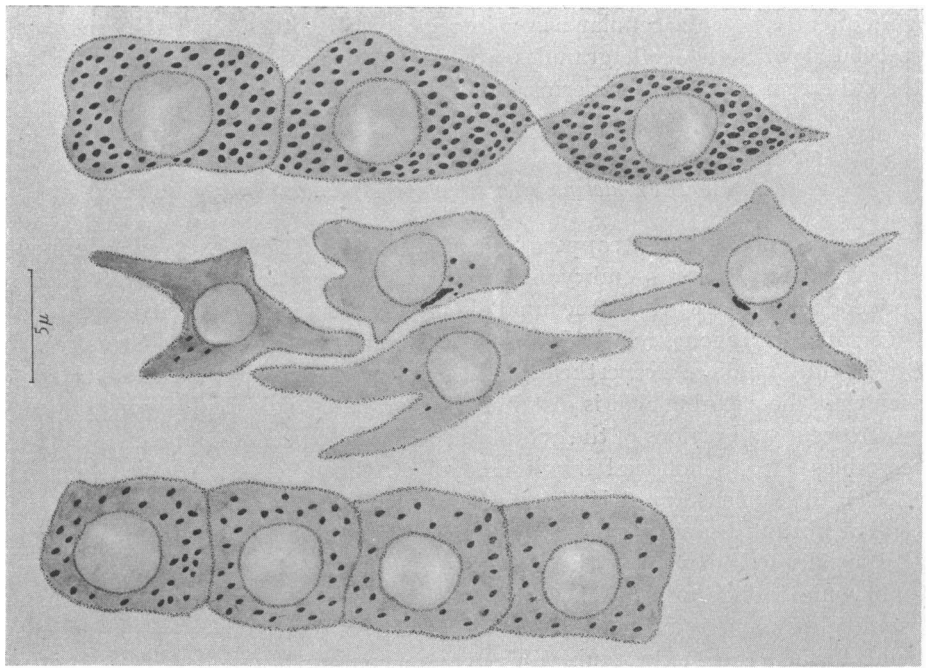
The abrupt disappearance of ascorbic acid at the boundary of the embryo is especially well marked in the ectoderm of embryos of from 1 to 3 days. The embryonic endoderm is often found to contain some ascorbic acid, though usually less than is present outside the embryo. The amnion and chorion, being formed from extra-embryonic tissues, contain heavy deposits. It has been found, however, that in 3-day embryos the deposit is absent from both layers immediately dorsal to the embryo. This phenomenon, which is not easily explained, may be compared with the absence of ascorbic acid from the yolk below the centre of some early blastoderms.

C. *The embryo after 4 days' incubation*

As we have seen, Ray (1934) was unable to find titrable amounts of ascorbic acid in chick embryos until the end of the fourth day of incubation. It is reasonable to suppose that the ascorbic acid demonstrated by the silver nitrate method in the early extra-embryonic tissues is present in too small quantity to be detected by the ordinary methods of quantitative analysis. At the fourth day, as might be expected from Ray's results, deposits of ascorbic acid appear

in a variety of tissues within the embryo. These include various parts of the central nervous system, part of the gut wall, some of the somitic material and certain mesenchymatous cells.

A striking and exceedingly constant phenomenon in the central nervous system is the dense impregnation of the dorsal ependymal layer at all levels (Pl. 1, fig. 11), and the heavy deposit which fills the thin roof-plate of the medulla; in the spinal cord, too, it is found that the inner, or ependymal, layer

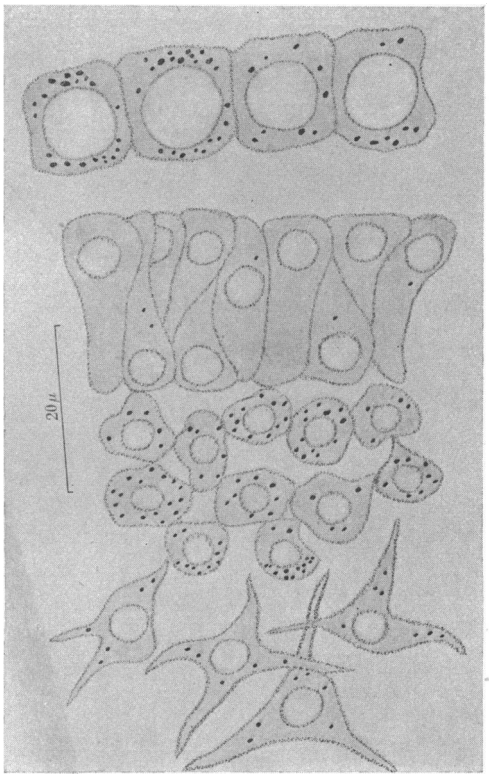


Text-fig. 4. Extra-embryonic cells of a 36 hr. embryo.

of the roof-plate is more heavily impregnated than other regions. The appearance of the medullary roof-plate is similar to that of the extra-embryonic ectoderm already described: that is to say, the cytoplasm of each cell is packed with spherical granules resembling mitochondria. On the other hand, the impregnation of the roof-plate in the spinal cord is unusual in being mainly on the cell membranes: relatively little of the black precipitate is within the cells (Pl. 1, fig. 12).

The other parts of the brain which, at this stage, contain conspicuous quantities of ascorbic acid, are the diencephalon and midbrain. In the first of these, in addition to a dense granular deposition in the inner layer of the roof, there are fairly numerous intracellular granules scattered through the whole depth

of the lateral walls; some of these granules adhere to the nuclear membrane, but many are distributed at random in the cytoplasm. A further phenomenon occurring in the diencephalon is the presence of ascorbic acid in the developing axons of some of the cells (Text-fig. 6); at this stage the cells concerned are confined to the ventrolateral region of the mantle layer. The pineal organ, which is represented by a small diverticulum, also contains ascorbic acid; the heaviest deposit is in the ependymal layer of the roof. Impregnation of



Text-fig. 5. Somitic cells of 4-day embryo.

developing axons can be observed in the midbrain and in the lateral walls of the spinal cord; in the latter this phenomenon is relatively inconspicuous. In the midbrain ascorbic acid also occurs dispersed in the body of the nerve cells. This is the case, too, in the ventrolateral part of the wall of the diencephalon, where the cells contain the highest concentration to be seen in the nerve-cells of the 4-day embryo.

The rudiments of the organs of the alimentary canal have at this stage, for the most part, little ascorbic acid. However, the liver and pancreas diverticula show a relatively heavy granular deposit, although histologically no difference

appears between their cells and the cells of the gut wall from which they are derived.

The distribution of ascorbic acid in the somites is indicated in Text-fig. 5. The dermatome cells contain almost none. In the myotome ascorbic acid is present in relatively large amounts, and can be seen outside the cells, attached to their processes, as well as in their cytoplasm; this is in accordance with the observation of Tonutti (1939) on developing muscle fibres, and is of interest since adult muscle is known to contain very little ascorbic acid indeed (Giroud, 1938, p. 69); moreover we have found that at a slightly later stage embryonic muscle contains no ascorbic acid which can be detected histochemically. The sclerotome at this stage contains less than the myotome; this confirms the preliminary report published by van Weel (1939).

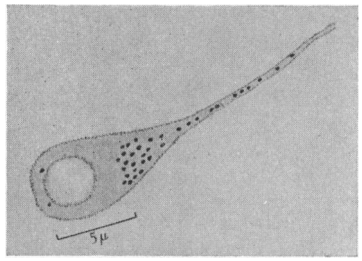


Fig. 6.

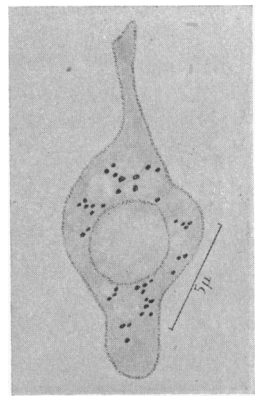


Fig. 7.

Text-fig. 6. Neurone from spinal cord of 4-day embryo, with ascorbic acid in axon.

Text-fig. 7. Cell containing fat (?) from mesenchymal tissue of 4-day embryo. Note ascorbic acid granules. See text.

In the neighbourhood of the dorsal root ganglia, and especially ventral to them, there are small groups of cells, distinct from those of the sclerotome and containing more ascorbic acid (Pl. 1, fig. 10). These cells seem to be the neural crest derivatives described by His (1892) as migrating ventrally in order to take part in the formation of the definitive sympathetic chain.

Two further types of cell remain to be described. Near the neural tube, in the undifferentiated mesenchyme which surrounds it, there are a very few, irregularly scattered cells which are quite distinct in appearance from their neighbours: they are highly refractile, owing to the presence in their cytoplasm of granules which have the appearance in the unstained preparations of fat droplets (Text-fig. 7). These cells cannot be distinguished in sections which have been stained with haematoxylin and eosin. Their interest lies in the fact that they contain ascorbic acid in association with the refractile material. In

addition to these very distinctive cells there are occasional cells indistinguishable from those of undifferentiated mesenchyme, except for the presence of ascorbic acid in granules attached to the surface of the nucleus.

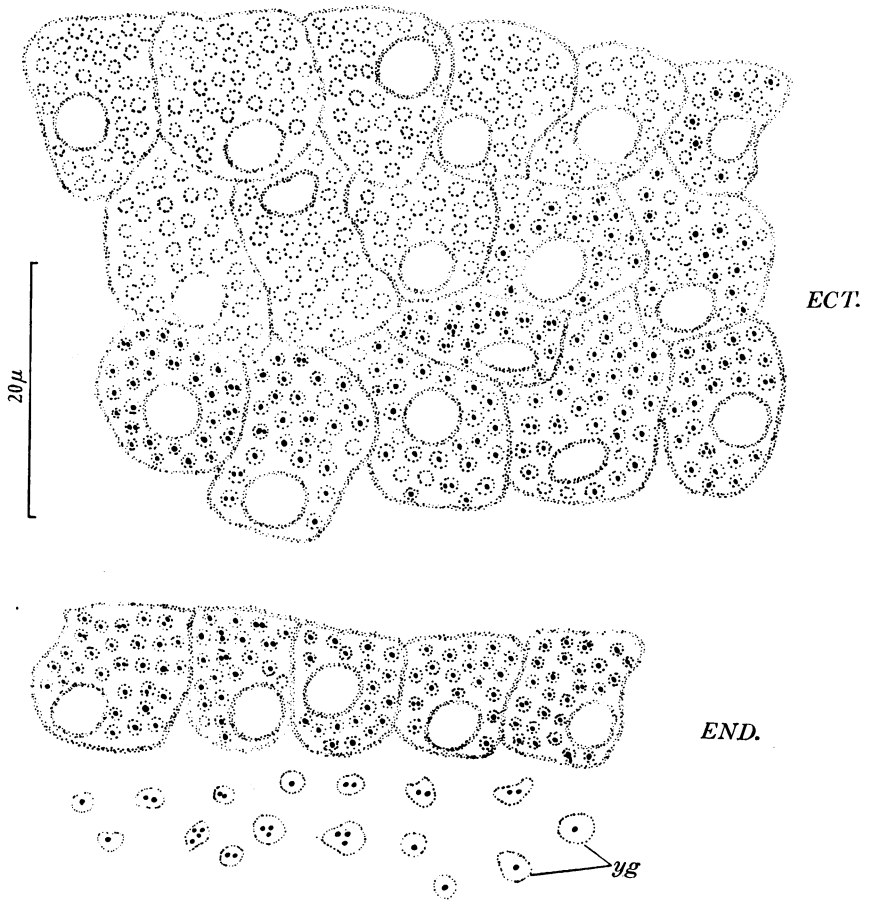
4. DISCUSSION

The association observed, of ascorbic acid with yolk granules, raises the question of its synthesis. There is very little information available regarding the mechanism of the synthesis of ascorbic acid in vivo. There is evidence that, as might be expected from its molecular structure, it is synthesized in plants from hexose precursors: Sah (1933) has put forward a theory of its formation from them in plant tissues. Guha & Ghosh (1933*a*, *b*) have brought evidence that mannose is a precursor of ascorbic acid in both animals and plants. The experiments of these authors with animal tissues have been repeated with negative results by Laporta & Rinaldi (1935), Scheunert & Schieblich (1937) and by Hawthorne & Harrison (1937). It therefore cannot be said that there is satisfactory evidence for synthesis from carbohydrates in animals.

Musulin *et al.* (1938, 1939) found that unsaponifiable fractions of halibut liver oil, oat oil and other vegetable oils, administered to rats, caused a marked increase in the rate of synthesis of ascorbic acid; these authors therefore suggested a lipoid precursor for the vitamin. Yolk granules contain a high proportion of lipoids, and the association observed between them and ascorbic acid in early embryos might be regarded as pointing towards synthesis from lipoids. The fact that the special cells, described above as apparently containing a large reserve of fat, also have ascorbic acid in association with it, is at least consistent with this suggestion. It may be added that the intracellular distribution of ascorbic acid observed in the extra-embryonic tissues does not conflict with this view: it is well established that ascorbic acid is associated with the "chondriome" in all cells which contain a high concentration of it (Bourne, 1935*b*; Giroud, 1938, p. 27); and though the mitochondrial system doubtless varies greatly in different types of cell, it has been shown to contain fats (Bensley & Hoerr, 1934). However, Musulin and co-workers, in continuing their investigations, have found that a wide variety of substances, including terpene derivatives and a number of aliphatic compounds, have an effect similar to that of fractions of animal and plant oils. These further results suggest that the substances used operate not as precursors, but by the indirect stimulation of the synthesis of ascorbic acid (Longnecker *et al.* 1939).

Although nothing can be concluded regarding the chemistry of the production of ascorbic acid, it is clear that the organs engaged in synthesis up to the end of the third day are the extra-embryonic tissues and the yolk granules below the blastoderm. Glycogen is already known to be laid down extra-embryonically until the liver becomes functional. The concept of an extra-embryonic "transitory liver", based on the latter fact, is thus strengthened by the observed distribution of ascorbic acid (see Needham, 1934).

The absence of ascorbic acid from the embryonic tissues during the first 3 days, and its sudden appearance in several tissues during the fourth, raise the question of the precise role of ascorbic acid in early development. There is little in any of the previous work on the physiology of vitamin C to provide guidance on this question. There is only one group of developmental processes in animals



Text-fig. 8. Semi-diagrammatic representation of part of the unincubated blastoderm photographed in Pl. 1, fig. 9, showing intra- and extracellular yolk globules (*yg*), some of them showing the presence of ascorbic acid.

in which it is certain that ascorbic acid plays an essential part: these are the laying down of the intercellular materials of connective tissue and bone (see Dalldorf, 1939, p. 339). There is extensive evidence, summarized by Giroud (1938, pp. 152 *et seq.*), that in flowering plants the presence of ascorbic acid is essential for the rapid growth of embryonic tissues, but no comparable observations have been carried out on animal material. The authors have found that chick heart fibroblasts and osteogenetic cells give little or no reaction

when growing rapidly in a medium containing an optimal amount of embryo extract (Willmer & Jacoby, 1936). Adrenal cortical and medullary cells from chicks of 17 days' incubation were also cultured in these conditions, with the same results. It is therefore clear that the presence of quantities of ascorbic acid in the reduced state is not necessary for the rapid growth of animal tissues.

It might be supposed, on the basis of the observations described above, that ascorbic acid plays no part in the development of the chick until the special processes of histodifferentiation have begun, but this does not account for its presence in high concentration in the extra-embryonic tissues. Perhaps the most likely explanation of the facts is that ascorbic acid is utilized by the early embryonic tissues, but that the oxidation potential which prevails in them is so high that ascorbic acid is oxidized immediately on reaching them. In the preparations of embryos of from 1 to 3 days there is usually a very faint black precipitate on the inner walls of the blood vessels, and of the lumina of the coelomic cavities and the neural tube; this may be due to the passage of ascorbic acid in the plasma and tissue fluids, from the extra-embryonic tissues into the embryo.

5. SUMMARY

1. A study has been made of the distribution of ascorbic acid (vitamin C) in the cells and tissues of chick embryos, from the unincubated blastoderm to the end of the fourth day of incubation.

2. The acid silver nitrate method was used for the demonstration of ascorbic acid. The validity of this method is discussed.

3. In unincubated blastoderms, and in those of 12 and 18 hours' incubation, evidence of ascorbic acid was found in association with yolk granules both within and outside the cells. The cells seen to contain ascorbic acid were confined to the periphery of the blastoderm.

4. In embryos of from 1 to 3 days the acid silver nitrate reaction shows the presence of ascorbic acid only in the extra-embryonic tissues, in particular in the ectoderm and endoderm. The cells of these layers contain large quantities of it dispersed in granules throughout the cytoplasm.

5. During the fourth day the silver nitrate reaction indicates the presence of ascorbic acid in the central nervous system, the liver and pancreas diverticula, and some mesoderm derivatives including the myotome. Its distribution at this age is described in detail.

6. The results are discussed with reference to the mechanism of synthesis of ascorbic acid in vivo, and to its role in development.

The authors are indebted to Prof. W. E. Le Gros Clark for his advice and for giving them the facilities of his department, and to Mr J. Z. Young and Dr R. B. Fisher for making suggestions. They also wish to thank Mr W. Chesterman for the care which he has taken with the photographs.

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EXPLANATION OF PLATE 1

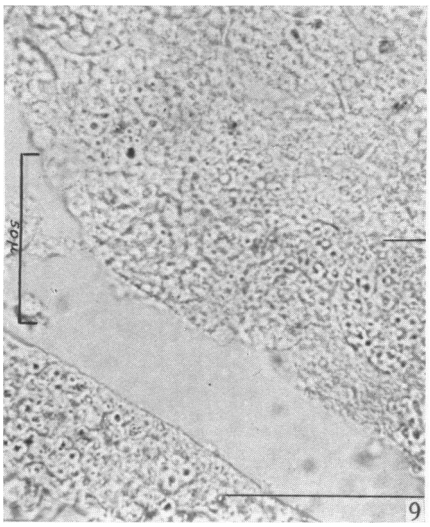
Photographs of unstained sections cut at 5μ

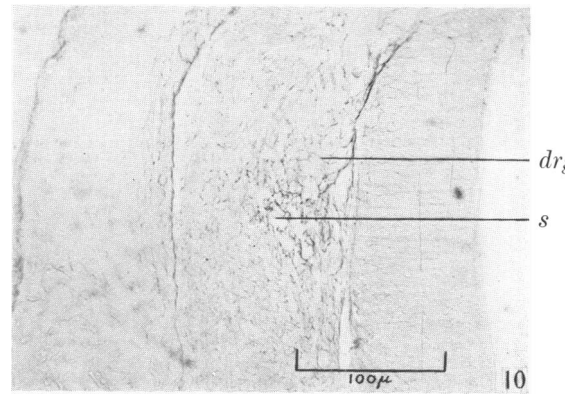
Fig. 9. (See also Text-fig. 8.) Unincubated blastoderm showing yolk globules with black precipitate; *ep* = epiblast, *en* = endoderm. Transverse section.

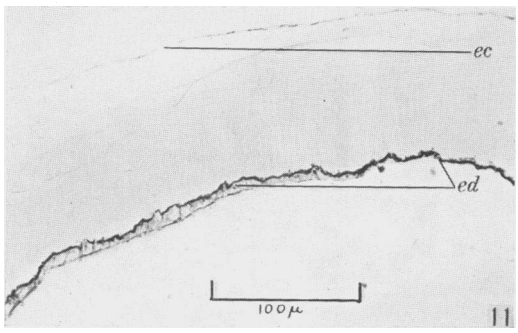
Fig. 10. Transverse section of 4-day embryo showing dorsal root ganglion (*drg*) and sympathetic cells containing ascorbic acid (*s*).

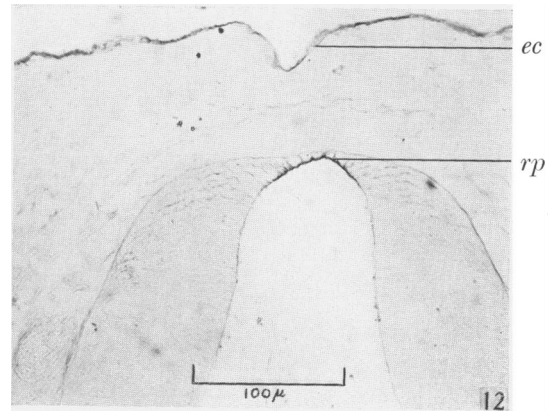
Fig. 11. Transverse section of 4-day embryo showing impregnation of dorsal ependymal layer of diencephalon (*ed*); *ec* = ectoderm.

Fig. 12. Transverse section of 4-day embryo showing impregnation of roof-plate of spinal cord; *rp* = roof-plate, *ec* = ectoderm.


50μ
ep
en
9


drg
s
100μ
10


ec
ed
100μ
11


ec
rp
100μ
12