Growth and Ascorbic Acid Content of the Chick Embryo

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ASCORBIC acid (ASA) is actively synthesized by germinating plant seeds (see Mapson, 1953), and by the embryos of various animal species (refs. in Needham, 1942).

Hauge & Garrick (quoted by Needham, 1931) found no ASA in the unincubated hen's egg. This was confirmed by Ray (1934), who showed that the vitamin C content of the chick embryo increases gradually after incubation of the egg. Since the egg is a closed system, it follows that the chick embryo can synthesize its own ASA and that the ASA content of the embryo at any given stage must be the balance between synthesis and utilization.

It was, therefore, considered of interest to make daily weighings and ASA estimations throughout development with the more sensitive methods now available in order to examine the possible relations between embryonic weight and ASA content on the one hand, and between growth rate and ascorbic acid concentration on the other. ASA concentration was also measured on some organs of early and late embryos and of adult birds.

MATERIAL AND METHODS

Growth measurements

Daily weighings were made on Light Sussex chick embryos from the second day of incubation till hatching.

Special precautions were taken in weighing early embryos to avoid errors due to evaporation and to prevent oxidation or loss of ASA in solution. The area pellucida was freed from the yolk and transferred to a hollow-ground slide inside a moist chamber, where the embryos were quickly but thoroughly freed from all extra-embryonic material, without using saline. The embryos were kept chilled in a stoppered weighing bottle, and before the last specimen was collected the container was wiped dry and its temperature was allowed to equilibrate inside the balance case. As soon as the last embryo was dissected, the pooled specimens were weighed in a semi-automatic balance. Stainless steel

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[J. Embryol. exp. Morph. Vol. 8, Part 4, pp. 527–39, December 1960]

instruments were used to prevent catalytic oxidation of ASA by traces of iron or copper.

The dry weight was calculated from the wet weight according to Murray's figures of percentage water content, quoted by Needham (1931). It may be noted that the embryo becomes dryer as it develops, but dehydration is not linear; the fastest rate is from the 11th to the 15th day. The average daily variation in water content is less than 1 per cent. of the embryo's weight, and therefore the error involved in calculating the water content should be negligible, even allowing for some difference between the material used here and that of Murray.

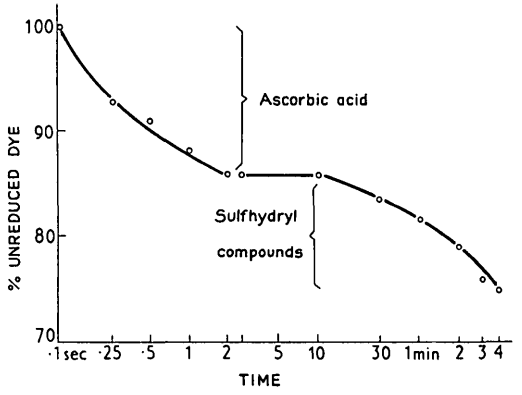
Several samples were weighed and analysed for each day of development. At the early stages, when the embryos were small, several specimens were pooled in each sample to decrease the experimental error.

Vitamin C estimations

The vitamin C estimations were made simultaneously with the growth measurements on the same material. Extraction of the vitamin with 5 per cent. metaphosphoric acid was done immediately after weighing the specimens. For small samples—early embryos or organs—the acid was added directly into the weighing bottle. Otherwise the specimens were ground with acid-washed sand and HPO_3 in a mortar.

Two methods were used for the vitamin C estimations: visual titration with 2,6-dichlorophenolindophenol according to the technique of Birch, Harris, & Ray (1933) and osazone formation with 2,4-dinitrophenylhydrazine (Roe & Kuether, 1943; Roe & Oesterling, 1944) as modified by Bolin & Book (1947). All solutions were made up with copper-free glass-distilled water and the dye solution was regularly standardized against pure crystalline ASA.

For dye titration the filtered extracts were adjusted to contain approximately 50 γ of ASA per ml. Below this concentration the accuracy of the method declines steeply. This method was used only when enough filtrate could be obtained for making triplicate titrations.



TEXT-FIG. 1. Continuous flow filtration of ASA from chick embryos.

The specificity of the dichlorophenolindophenol method is satisfactory for animal tissues, where the few substances known to interfere within the same range of redox potentials at pH lower than 5, such as glutathione or cysteine, reduce the dye at a much slower rate than ASA, so that interference can be avoided by carrying out the titration in less than 30 seconds (Harris *et al.*, 1948). The specificity in chick embryonic material was checked by the 'continuous flow' method of Harris & Mapson (1947). Text-fig. 1 shows the amount of dye consumed during the first 2·5 seconds by a sample extract of chick embryos titrated in the flow apparatus at pH 4·5. This was equivalent to 69 γ of ASA/ml., exactly the same titre as that obtained by the visual titration technique in 30 seconds. The second part of the curve is similar to the calibration curves obtained with reduced glutathione or cysteine.

The dinitrophenylhydrazine method was used exclusively for all estimations in early embryos and individual organs when the sensitivity of the oxidation-reduction method was not sufficient, and also in later stages as a complement and a check to dye titrations. The osazone method is some fifty times more sensitive than the visual titration method and it has the further advantage that oxidized ASA also can be estimated. Reducing substances occurring in embryonic tissues, such as sulphydryl compounds, do not interfere, but glucuronic acid and some of the precursors of ASA may. Thus, with the possible exception of reductones that are not present in fresh animal tissues, there is no overlapping of interfering substances between this and the dye titration method, so that both could be used together for testing the specificity of ASA estimations. With freshly obtained embryonic tissues, the two methods agreed within 10 per cent.

Bolin & Book's modification of Roe's method was found to have several advantages. Firstly, less filtrate is required; secondly, the risk of loss of vitamin by adsorption on charcoal is eliminated; and thirdly, the colour remaining after adding a drop of dye to the solution gives a rough indication of its titre and shows whether further dilution is necessary. The technique was standardized with pure ASA (Roche) oxidized with bromine, according to Roe's original procedure. Almost perfect agreement was obtained between aliquots with bromine and with dichlorophenolindophenol. The colour obtained with dinitrophenylhydrazine was found to be stable for 48 hours at 4° C., so that the readings could be rechecked when necessary. A blank was obtained for every sample analysed, and reagent blanks were also run frequently. All samples were suitably diluted to fall within the linear range of the method, which was from 0·5 to 10 γ of ASA/ml.

RESULTS

Growth measurements

The results of daily weighings are shown in Table 1 and in Text-fig. 2. It may be noticed that the curves obtained for both wet and dry weight are sigmoid.

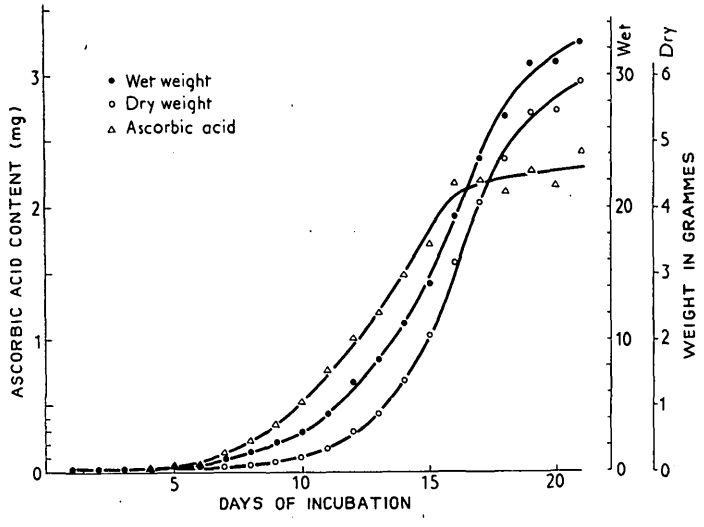
The coefficient of variation showed only small fluctuations along the curve, except for the 3rd day.

TABLE 1

Weight of chick embryos

Figures in brackets indicate weight with 'spare yolk' included (see text).

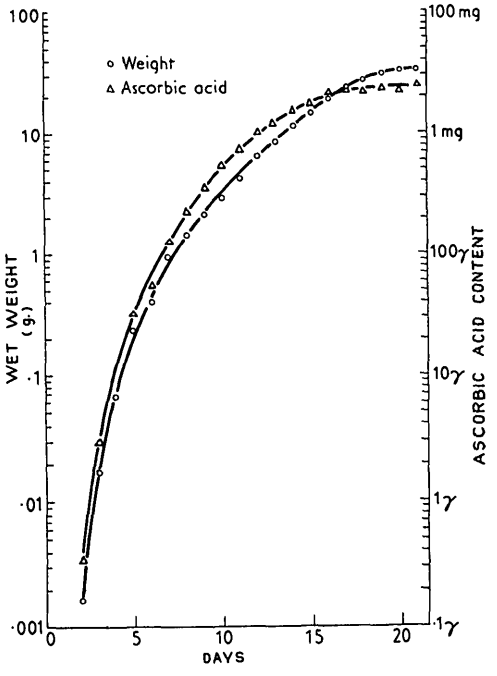
Days	*Number of embryos*	*Number of samples*	*Average wet weight per embryo (g.)*	*Coefficient of variation*	*Average dry weight per embryo (g.)*	*Growth rate (dw/dt)*	*Specific growth rate (1/w) (dw/dt)*
2	13	3	0·00162	0·002	0·000075	..	..
3	31	4	0·0169	2·516	0·0008	..	..
4	10	3	0·0665	0·605	0·0033	0·107	1·61
5	14	4	0·227	0·501	0·012	0·144	0·63
6	14	7	0·397	0·084	0·023	0·359	0·90
7	6	4	0·935	0·011	0·055	0·525	0·56
8	3	3	1·418	0·000	0·086	0·571	0·40
9	4	4	2·094	0·004	0·136	0·654	0·31
10	5	3	2·812	0·054	0·197	0·993	0·35
11	5	3	4·241	0·006	0·318	2·043	0·48
12	4	3	6·670	0·003	0·587	2·107	0·32
13	4	3	8·443	0·002	0·852	2·167	0·26
14	2	2	11·16	0·005	1·36	2·769	0·25
15	6	5	14·18	0·007	2·05	4·184	0·30
16	8	6	19·34	0·017	3·18	5·008	0·26
17	7	6	23·65	0·018	4·07	3·59	0·15
18	4	3	26·82	0·000	4·72	3·89	0·15
19	4	4	30·94	0·003	5·48	2·05	0·07
20	4	4	31·00 (39·51)	0·004	5·60	..	..
21	6	6	32·55 (45·20)	0·002	5·90	..	..



TEXT-FIG. 2. Wet and dry weight and ASA content of chick embryos.

As is well known, the yolk sac is incorporated into the abdominal cavity before hatching, usually after the 19th day of development. This causes a sudden increase in weight, and it is debatable whether the added mass contributed by the extra-embryonic structures should be computed as embryonic weight or whether it should be excised before weighing the embryo.

The incorporation of the yolk sac may be one of the reasons why some workers (see Needham, 1931) have missed the flattening of the curve at the end of development; their figures give the wrong impression that the growth-curve of the chick embryo is exponential, and not sigmoid.



TEXT-FIG. 3. Wet weight and ASA content per embryo (semi-log. plotting).

The weight-curve has a point of inflexion between the 15th and 17th days which is in close agreement with the findings of Lamson & Edmund, Hasselbalch, and Schmalhausen (see Needham, 1931). Consequently, the peak of the first derivative dw/dt, or velocity curve, is at the 16th day as shown in Table 1. (In this and all the following equations w stands for weight and t for time.)

The semi-log. plotting (Text-fig. 3) shows a hyperbola concave to the abscissa, emphasizing the fact that the specific growth rate is highest at the beginning and decreases continuously throughout development. The 'specific growth rate' $(1/w)\,(dw/dt)$ (Table 1) plotted against time approximates an inverted hyperbola which shows a deceleration with time.

An attempt to find a reasonably simple equation which would fit the data

approximately indicated that the first portion of the curve was fitted by a Gompertz equation while the middle and late parts were better fitted by a logistic curve.

Vitamin C estimations

The negative findings of earlier workers for the yolk and the white of unincubated eggs were confirmed in the present experiments by means of the more sensitive phenylhydrazine method. Even with this method it is not possible to rule out the presence of traces of reduced or oxidized vitamin smaller than 0·5 γ per ml. in the white or the yolk, but as the eggs weighed approximately 50 g. without the shell, they could not contain more than 25 γ at most before incubation, while the embryos were found to contain more than 30 γ at the 5th day of incubation, and nearly 2·5 mg. at hatching. One may conclude, therefore, that the chick embryo actively synthesizes ASA.

TABLE 2

Ascorbic acid content of chick embryos

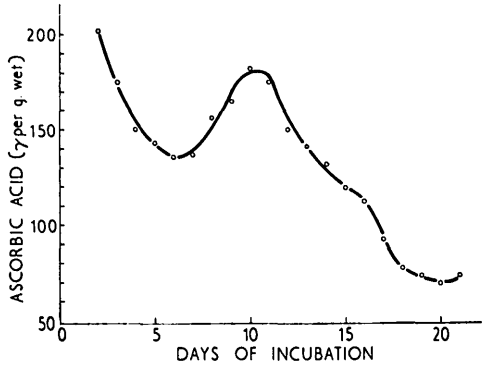
Days	*Average ASA content per embryo*	*Coefficient of variation*	*Average ASA content per g. wet weight*	*Coefficient of variation*	*Average ASA content per mg. dry weight*
2	0·33 γ	0·011	204 γ	0·069	4·0 γ
3	2·95	3·597	175	0·044	3·7
4	10	0·050	150	0·486	3·3
5	32	0·246	143	0·114	2·7
6	54	0·271	136	0·140	2·3
7	128	0·048	137	0·012	2·3
8	222	0·015	156	0·016	2·6
9	345	0·003	165	0·013	2·5
10	514	0·170	182	0·023	2·6
11	752	0·017	175	0·000	2·4
12	1,000	0·011	150	0·003	1·7
13	1·19 mg.	0·003	141	0·000	1·4
14	1·48	0·005	132	0·000	1·1
15	1·71	0·032	120	0·012	0·83
16	2·19	0·030	113	0·025	0·69
17	2·20	0·006	93	0·018	0·54
18	2·11	0·036	78	0·012	0·45
19	2·27	0·038	74	0·063	0·41
20	2·16	0·008	70	0·003	0·39
21	2·42	0·000	74	0·008	0·41

Vitamin C appears very early in the embryo. A positive reaction was obtained with Bolin & Book's procedure on 24-hour blastoderms, and at 48 hours the embryos already contained 0·3 γ on an average.

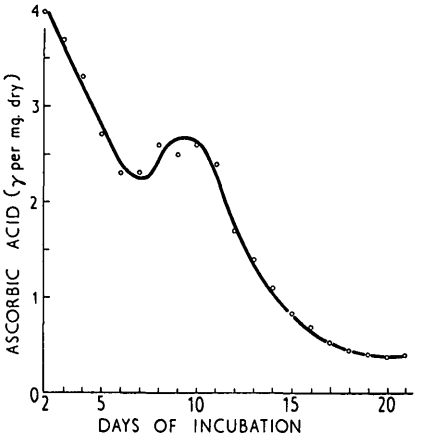
The results of daily estimations of ASA in whole embryos are shown in Table 2 and in Text-figs. 2 and 3. It may be noticed that there is a high correlation between the ASA content of the embryo and its weight up to the point of inflexion of the weight-curve (16th day), at which point the vitamin content

ceases to increase altogether. The change in ASA content per embryo showed a fairly good approximation to a logistic curve.

The relative increase in vitamin in respect to the increase in total embryonic mass was calculated with Huxley's (1932) formula $y = bx^k$, where b is a constant, y is the magnitude of the part, in this case vitamin C, x the magnitude of the whole, in this case embryonic weight, and k is the 'heterauxetic constant'. The double logarithmic plotting resulted in a series of straight lines, but instead of quoting different heterauxetic constants for different stages of development, it was preferred to find the nearest linear approximation by the least square method and to give a single k which can be compared with that of other compounds. The value of k thus obtained was 0·896.



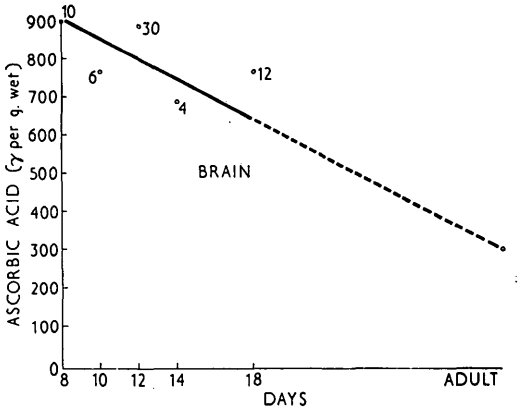
TEXT-FIG. 4. Concentration of ASA per gram of wet tissue.



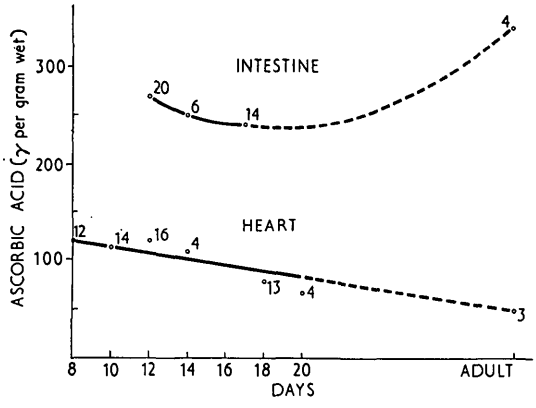
TEXT-FIG. 5. Concentration of ASA per gram of dry tissue.

The concentration of ASA per gram of wet tissue is shown in Text-fig. 4, and that per gram of dry tissue in Text-fig. 5. From the 2nd day of development until hatching the ASA concentration showed a decline from 204 γ to 74 γ per g. of wet embryo and from 4·0 to 0·41 γ per mg. dry embryo. For dry tissue, therefore, the decline is of the same order as that of the growth rate, i.e. to about 1/10 of the initial value. For wet tissue, on the other hand, the growth rate falls to about 1/8 while the ASA concentration declines to only about 1/3. Moreover, the curves of specific growth rate and of ASA concentration are not parallel. This discourages the suggestion of a simple correlation between the concentration of vitamin C and the rate of cell-division in the embryo.

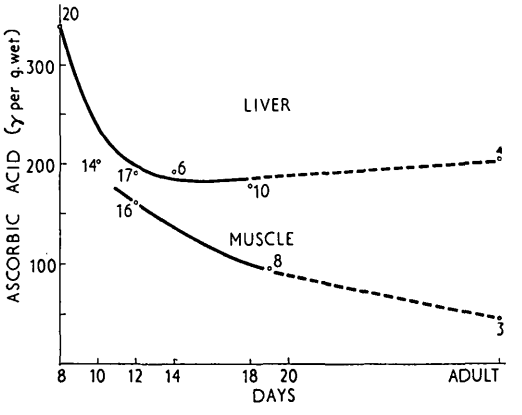
The technical obstacles to making reliable estimations on individual organs at the early stages of development are even greater than with whole embryos, and therefore no values are given before the 8th day of incubation. The results obtained with brain, heart, skeletal muscle, liver, and intestine are shown in Text-figs. 6, 7, and 8. The general trend is for the concentration of ASA to fall



TEXT-FIG. 6. ASA concentration in brain of chick embryo.



TEXT-FIG. 7. ASA concentration in intestine and heart of chick embryo.



TEXT-FIG. 8. ASA concentration in liver and muscle of chick embryo.

with age, particularly when the embryonic values are compared with those obtained in the organs of the adult cock. The fall in ASA from embryonic to adult life was most marked in brain and muscle. The ASA concentration in the embryonic liver decreased substantially from the 8th to the 10th day, and thereafter it remained approximately at the adult level, while the intestine showed a slightly higher concentration in the adult bird than in the embryo.

Measurable amounts of ASA appear in the yolk sac at the later stages (Table 3). This may have been either synthesized or filtered back by the sac villi.

TABLE 3

ASA concentration in the yolk sac

Days of incubation	*Reduced ASA (γ/g.)*
16	14
17	19
18	18
19	23
21	19

The ratio of oxidized/reduced vitamin in the whole embryo increased with age. During the first week the percentage of oxidized vitamin oscillated between 0 and 4 per cent. of the 'total' vitamin estimated by Bolin & Book's method, while at the 18th and 19th days as much as 29–33 per cent. was extracted in the oxidized forms (dehydroascorbic plus diketogulonic acid). This was probably due to the increase in the blood-volume of the embryo, since a large proportion of the circulating vitamin is usually extracted in the oxidized state because of its interaction with oxyhaemoglobin (Butler & Cushmann, 1940; Roe & Kuether, 1943). Individual organs of late embryos and of adult birds drained of blood contained negligible proportions of oxidized vitamin, in accordance with the experience of various workers on other animal species.

DISCUSSION

The contention of Ray (1934) that ASA is absent until the 4th day of incubation must be attributed to the low sensitivity of the methods then available, since at least a hundred embryos would be necessary to obtain a clear reading with the visual titration method at the 3rd day of incubation. Barnett & Bourne (1942) also failed to find any ASA in the embryo before the 4th day with a histochemical test based on the reduction of silver nitrate, but Weel (1948), using Bourne's method, obtained a positive reaction in the limb-bud at the 3rd day.

The vitamin content per embryo ceases to increase at the 16th day, coinciding with the inflexion of the weight curve. The evolution of CO_2 per egg also becomes stable at this date according to the figures of Pott & Preyer and of Bohr and Hasselbalch quoted by Needham (1931).

The concentration of ASA per gram of wet and dry tissue is highest at the beginning of development coinciding with the maximum specific growth rate and shows a hump towards the middle of development, between the 7th and the 12th day, which could be related to any of a number of important metabolic events which take place at this stage, namely, a peak in the metabolic rate and in the ratio of burnt to stored solids, a change-over from carbohydrate to fat consumption accompanied by a peak in the lipase activity of the yolk sac, a peak in free glucose and the onset of glycogen storage and insulin secretion (refs. in Needham, 1931, 1942). It is noteworthy in this connexion that ASA catalyses the oxidation of some fatty acids and lipids and that lipase activity is decreased in scurvy (refs. in Reid, 1954). Insulin secretion also falls steeply in scorbutic guinea-pigs (Bannerjee, 1944). A correlation between general metabolic activity and ASA concentration has also been found by Bessey & King (1933) in animals and by others in plants (refs. in Mapson, 1953).

The heterauxetic constant found for ASA in the embryo is almost identical with that obtained by others for glutathione (GSH) (data in Needham, 1942). Needham's (1942) cautious remark that 'it is doubtful whether ascorbic acid shows the bradyauxesis of the other highly reducing substance, glutathione' reflects the lack of sufficient and accurate estimations at the time. Early in his work on GSH, Hopkins (1921) found that this compound was missing from the white and yolk of the egg, but the chick blastoderm gave a positive nitroprusside test. This suggests that ASA and GSH appear simultaneously in the embryo from the beginning of development. ASA and GSH also appear simultaneously in germinating seeds and sprouting tubers (see Mapson, 1953).

The curve of GSH concentration per unit of dry weight obtained by Murray (1926) in the chick embryo overlaps closely with the corresponding portion of the ASA concentration curve (Text-fig. 5). The relationship between these two compounds is of interest because of the protective action of GSH on ASA, the possible occurrence in animal tissues of a respiratory pathway (Ames & Elvehjem, 1946) like that described by Mapson & Goddard (1951) in plant tissues, and the role assigned to SH groups in cell-division by Rapkine and others (discussion in Needham, 1942; Swann, 1957).

The results obtained with isolated organs confirm the findings of Bessey & King (1933) that the organs of rapidly growing animals show higher vitamin titres than those of older individuals. The values given by these authors for the adult fowl are in good agreement with the present figures. The steepest fall in ASA with age was found in the two tissues which cease to divide in adult life, namely, brain and muscle. On the other hand, the intestine, which keeps its embryonic level of ASA in postnatal life, has a high mitotic rate and a fast turnover of DNA in the adult organism (Hevesy & Ottesen, 1943).

The stability of ASA concentration in the liver after the 10th day agrees with the work of Ray (1934), who found steady values around 270 γ per g. wet weight in late development. The decrease in liver ASA from the 8th to the 10th day

found in the present experiments may be significant in connexion with the onset of glycogen storage in this organ, which takes place approximately at the 8th day (refs. in Needham, 1942). Barnett & Bourne (1942), using Bourne's histochemical silver method, could locate ASA in the liver up to the 10th day, but found 'none at all' after that date. ASA appears to influence glycogen storage and synthesis (see Reid, 1954), while ASA deficiency causes a marked decrease in liver phosphorylase (Murray, 1950).

According to Olivo & Porta (1931) the mitotic index of the liver falls from 14·3 to 7·73 from the 7th to the 10th day, which represents a decline of about 45 per cent., and is therefore approximately equivalent to the fall in ASA concentration for the same period. Here, however, the correlation ends, for the vitamin concentration remains constant after the 10th day, while the mitotic index continues to fall to about 1·0 at hatching. On the other hand, the specific growth rate $(1/w)\,(dw/dt)$ calculated from Olivo & Porta's figures falls only from about 0·8 to about 0·2 from the 7th to the 19th day. It may be noted that this is a less subjective and more accurate index of growth than the mitotic coefficient.

Similarly, there is no visible correlation between the pronounced fall in the mitotic index of the heart, which decreases nearly fifteen times from the 7th to the 21st day (Olivo & Porta, 1931), and the moderate fall in its ASA concentration.

Since no direct stimulation of cell-division by ASA has been definitely demonstrated in animal tissues and ASA is not a growth factor for most micro-organisms and protozoa, its marked influence on the growth of guinea-pigs and its active synthesis by rapidly growing tissues may perhaps be connected with the building of intercellular materials rather than with cell-growth and multiplication. It is now well established that ASA is necessary for the synthesis and maturation of reticulin, collagen, the bone matrix, and dentine, and there is good evidence that it also influences the metabolism of mucopolysaccharides (see review by Reid, 1954). Yet, in spite of the fact that most of the functions of ASA so far established are exerted on mesenchymal structures, epithelial and nervous tissues are richer in ASA than mesenchymal tissues in both the embryo and the adult.

It cannot be established from the present results whether the vitamin can be synthesized by all or only by some tissues of the embryo, nor whether the variations in ASA content at different sites and at different stages of development are due to variations in synthetic capacity or merely to differential storage. It is possible that the ability to synthesize ASA shown by the early embryo is not shared by all tissues in late development and that some cells may lose the synthetic mechanism in the course of differentiation.

SUMMARY AND CONCLUSIONS

Daily weighings of chick embryos of the Light Sussex strain gave a sigmoid curve with a point of inflexion at the 16th day. It was shown that inclusion of

the 'spare yolk' after the 19th day may give a misleading impression of fast growth at the end of development. The growth rate was greatest at the beginning of development and the specific growth rate decreased approximately as an inverted hyperbola with time.

No ascorbic acid was found in the unincubated egg, but it appeared in the blastoderm from the beginning of development coinciding with the appearance of glutathione. The total amount of vitamin per embryo gave a sigmoid curve with a maximum at the 16th day coinciding with the peak of the differential growth curve. The concentration of ASA per unit weight, however, like the specific growth rate, was maximal at the beginning of development; it showed a lower peak towards the 10th day which was not correlated with any significant variation in growth, although it may have been related to any of several metabolic changes that occur at this stage in the chick embryo.

ASA estimations in individual organs showed that, in general, epithelial tissues are richer in vitamin than mesenchymal tissues, and that in those tissues that divide very actively during embryonic development but cease to multiply in adult life, such as muscle and the central nervous system, the vitamin C concentration drops markedly with age. The brain showed a remarkably high concentration in early development.

It may be concluded that the changes in weight and in ASA content of the chick embryo both follow sigmoid curves, that the chick embryo is able to synthesize ASA from early stages of development, and that there is some correlation between the concentration of ASA and the rate of growth both in the whole embryo and in some of its organs.

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(Manuscript received 29:i:60)