PHARMACOLOGICAL STUDIES ON THE EMBRYONAL STAGE ON THE VITAMIN C METABOLISM OF THE DEVELOPING HEN'S EGGS*

SOHAKU SEKIYA

Department of Pharmacology, Showa Medical College, Tokyo

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INTRODUCTION

As part of the pharmacological studies of embryonal stage, Wada (1) of our Department investigated the influences of saccharin on the chick embryo, and confirmed that the "fertile egg injection method" is extremely advantageous to survey the activity and the fate of various substances in the living individuals. Employing this method, Wada conducted experiments concerning the toxicity of saccharin, its influences on the development of chick embryo, its diuretic activity, pathologic histologic investigation on various organs, and relation to the fate of saccharin and proteolytic enzymes of the liver, thereby he clarified the pattern of the excretion of saccharin. Further, Mori (2) performed a detailed investigation in the influences of *parotin* (a salivary gland hormone) on the development of chick embryo in general and on the development of bone of chick embryo, thereby he obtained an extremely interesting results.

When the author investigated the influences of various chemicals on the vitamin metabolism in the embryonal stage, this method was employed as it was done by Wada and Mori. As the first step, attempts were made to investigate the influences of various vitamins on the vitamin C metabolism in the embryonal stage. In the present experiment, the rise and fall of the vitamin C content in the course of incubation of fertile eggs without treatment was traced on the chick embryo as well as on various other portions of the eggs.

MATERIALS AND METHODS

Fertile eggs were obtained from a nearby poultry farm. They were laid by white leghorns. They were as fresh as 1—2 days old. Eggs of a roughly uniformed size (approximately 50 g in weight) were selected and incubated in an electrically regulated incubator at 38°C (103° F) and at the humidity of 64—65%. Utmost care was taken of the ventilation of the incubator. Eggs

*Director: Prof. Sh. Tsunoo

were taken out for experiments at various stages of development.

The primordium of the chick embryo generally appears approximately on the 3rd day of incubation. However, in the earlier stage, larger number of embryo is required for the estimation. Therefore, eggs were used for the experiment beginning from the 5th day of incubation when only small number of embryo is sufficient for the estimation.

During the developing stage of the chick embryo, the amniotic and allantoic fluids are clearly separable, and it is not too difficult task to separate without bymixture of other substances. Even so, procedures were conducted with great care. From the 6th day of incubation, the separation of allantoic fluid becomes easier, and it is thin fluid in the beginning, while it becomes slightly cloudy from approximately the 15th—17th day of incubation. Similarly to the allantoic fluid, the amniotic fluid was collected and employed for the experiments from the 6th day of incubation. Generally, the amniotic fluid is thin, but it becomes viscid in the later stage of the development.

First of all, the shell is broken in the middle of the egg, then the allantoic membrane which is holding the allantoic fluid is cut open to collect carefully the allantoic fluid without bymixture of other substance for experiment. The amniotic membrane is then cut open to collect amniotic fluid. Separately, part of the egg yolk is collected for the experiment. The eye balls of the embryo are next removed at the optic nerve bundle and employed for experiment separated from the embryo.

The former three (allantoic fluid, amniotic fluid and egg yolk) are quantitatively diluted to contain as high concentration as possible of vitamin C with metaphosphoric acid solution and distilled water. In the latter two cases (embryo and eye balls), the tissues are ground thoroughly and rapidly in a mortar with purified marine sand before they are diluted.

The estimation of vitamin C was conducted according to the indophenol method (3).

As to the calculation of day of incubation, the 1st day meant that the egg was placed in the incubator 24 hours or less ago. Thus, every 24 hours was counted 2nd, 3rd, and 4th day and so on.

The embryo on the 21st day means that it is 3 hours after a small hole was made by the embryo on the egg shell after the end of the 20th day of incubation. However, in this case, only the body weight was measured but the estimation of vitamin C was not conducted. Therefore, as soon as the time prescribed was attained, the egg was taken out from the incubator for the experiment.

Until the 5th to 10th day of incubation, materials from 3—5—10 eggs have to be pooled for the calculation of the estimated values of one group, because until such time, the weight of embryo and eye balls are too light and the

allantoic fluid and amniotic fluid are too scanty. In every experiment, the average value of 5—6 groups was calculated. After the 11th day of incubation, one group consisted of 1—3 eggs and the average value of 5 groups was calculated. However, for the eggs on the 5th day of incubation, the estimation was conducted only on the embryonal body. For the embryonal body and eye balls, the estimations were conducted until the 20th day, and on allantoic fluid, amniotic fluid and egg yolk until the 18th day of incubation.

RESULTS OF EXPERIMENTS

A. On the Embryonal Body Weight and Other Changes during the Incubation (See Table 1)

In the development of chick embryo, geometrically progressive increase in the body weight is generally seen in the last half of the incubation. For instance, the body weight on the 5th day is 0.4 g, whereas it is 2.8 g on the 10th day, showing an increase by approximately 7 times. The increase in the body weight after the 10th day is very sharp compared with that in the first half of the incubation. On the 18th day, the body weight is approximately 20 g, while on the 21st day, it is approximately 30 g, demonstrating an increase by approximately 10 g within the last three days.

The weight of the eye balls also increase in parallel with the increase in the weight of the embryonal body. In the first half of the incubation, it occupies $1/6$ to $1/5$ of the embryonal body, but it increases gradually during the course of development.

As to the changes in the general appearance, beginning from the 6th day, the cervical region becomes slender and the extremities begins to take forms. Also, the portion connecting the yolk sac and the embryo becomes thinner. In the embryo on the 8th day, the lower extremities looks markedly longer,

TABLE 1

Day of incubation	Egg count	Average weight of embryo (g)	Average weight of eye balls (g)	Average amount of allantoic fluid (g)
5	38	0.4	—	—
6	35	0.7	0.15	1.3
7	25	1.0	0.20	1.8
8	27	1.5	0.35	2.5
9	23	2.4	0.40	2.2
10	20	2.8	0.45	2.5
11	12	3.6	0.50	3.5
12	10	4.9	0.55	3.4
13	12	7.4	0.60	5.2
14	8	9.0	0.65	6.0
15	9	13.4	0.70	8.1

16	8	14.8	0.75	4.5
17	10	17.2	0.80	4.0
18	8	19.4	0.80	3.8
19	8	22.5	0.80	—
20	6	24.0	0.80	—

the upper and lower bills are well developed, and the so-called egg-tooth is also found developing. On the 11th day, the growth and differentiation of the four extremities becomes further evident showing that the upper extremities are taking the form of the wings and the lower extremities are taking the form of the legs. Roughly on the 12th day of incubation, feathers begin to grow on the skin.

Relation to the allantoic and amniotic fluid, the pattern of the changes were described in the above. The egg yolk is viscid in the earlier stage, but the viscosity decreases gradually in the later stage, and further, viscid portion and less viscid portion are found intermingled. The volume of egg yolk also decreases gradually, and almost no egg yolk is found in the terminal stage of the incubation.

B. On the Change in the Vitamin C Content during the Incubation

It has already been reported by numerous earlier workers that the egg yolk and egg albumen contains no vitamin C or very little. G. H. Satterfield *et al.* (4) (1947) recognized that, even when a hen was given a total of 2150 mg of *l*-ascorbic acid in 21 weeks by 2—4 subcutaneous injections per week, the eggs laid by the hen do not contain any amount of vitamin C.

I. On the vitamin C content of chick embryo during the incubation (See Table 2)

The total vitamin C content of the chick embryo increases beginning from the 5th day (body weight 0.4 g, vitamin C 15.19 mg%) up to the 12th day of incubation (body weight 4.9 g, vitamin C 29.52 mg%), and gradually decreases later to 15—16 mg%. On the 20th day of incubation, the body weight is 24.0 g containing 16.43 mg% of vitamin C.

The correlationship between the reduced vitamin C content and oxidized vitamin C content in the chick embryo demonstrates that the former is always larger than the latter. Similar to the total vitamin C content, the reduced vitamin C content increases up to the 12th day of incubation (25.41 mg%) and gradually decreases later. On the 20th day of incubation, it is 13.85 mg%.

The oxidized vitamin C content increases beginning from the 6th day of incubation (3.60 mg%) up to the 9th day (6.63 mg%), and then reaches the bottom on the 11th day (1.76 mg%). It gradually increases again up to the 15th day (9.93 mg%) and then decreases again. On the 20th day of incubation, it is 2.58 mg%.

II. On the vitamin C content of eye balls during the incubation (See Table 2)

As stated in the above, it is characteristic that the weight of the eye balls is relatively heavy in the earlier stage. The total vitamin C content gradually increases during the incubation up to the 9th day of incubation (15.81 mg%) and then gradually decreases. Thereafter, it increases up to the 12th day (16.81 mg%) again, and then decreases to the bottom on the 17th day (10.40 mg%). However, a slight increase is seen thereafter. In the relationship between the reduced vitamin C content and the oxidized vitamin C content, an equal value is seen in terms of mg% on or about the 8th day of incubation. But, up to approximately the 12th day thereafter, the reduced vitamin C content is larger than that of the oxidized vitamin C content. However, the oxidized vitamin C content becomes larger than that of the reduced vitamin C content at later date. Beginning from the 10th day (10.91 mg%), the reduced vitamin C content decreases to 3.5—4.5 mg%. Once on the 10th day, the oxidized vitamin C content decreases to 2.6—3.1 mg%, and thereafter, it increases gradually.

III. On the vitamin C content of allantoic fluid during the incubation (See Table 3)

The total vitamin C content of the allantoic fluid in terms of mg% increases as the incubation progresses. It reaches the peak on the 11th or 12th day of incubation (7.21—6.84 mg%), then after a transient decrease, it increases again. In the relationship between the reduced vitamin C content and the oxidized vitamin C content, beginning from the 9th day of incubation, the oxidized vitamin C content becomes larger in the last half of the incubation.

During the whole course of incubation, the reduced vitamin C content demonstrates no marked rise and fall. It maintains the level of 1.5—2.5 mg%.

The oxidized vitamin C content changes roughly in the same manner as that of the total vitamin C content showing an increase in terms of mg%.

IV. On the vitamin C content of amniotic fluid during the incubation (See Table 3)

During the whole course of incubation, the total vitamin C content maintains the level of 2.0—3.0 mg%. Further, in the relationship between the reduced vitamin C content and the oxidized vitamin C content, no remarkable difference is noted between the two. A slight increase is demonstrated in the reduced vitamin C content on about the 9th—16th day of incubation. Almost no fluctuation is noted in the oxidized vitamin C content during the whole course of incubation.

V. On the vitamin C content of egg yolk during the incubation (See Table 3)

The total vitamin C content increases gradually since the beginning of the incubation up to the 7th day (8.33 mg%) indicating that the egg yolk contains relatively large quantity of vitamin C. However, it gradually decreases in the last half of the incubation. Moreover most part of the total content is the oxidized vitamin C.

The reduced vitamin C content demonstrates no marked fluctuation during the whole course of incubation keeping the level of 0.5 — 0.8 mg%.

C. On the Influence of Blood Component Containing Tissues on the Estimated Values of Reduced Vitamin C Content

Generally speaking, in the cases of the tissues containing a large amount of blood component, the ascorbic acid is transformed to the oxidized vitamin C when the material is deproteinized with metaphosphoric acid, thus the value given as the reduced vitamin C content tends to be smaller than the actual value. Therefore, such influence was investigated on the materials from the embryo. For the purpose, one part of the material was deproteinized after the aeration with CO gas, and another part was deproteinized without such aeration. However, there were no difference in the values thus obtained. Consequently, in view of the above experiment, it is considered that influence of the blood component on the values of reduced vitamin C content obtained in the cases of the embryo, eye balls etc. was almost negligible.

TABLE 2. On the vitamin C content of chick embryo and eye balls during the incubation (mg%)

Portion Vitamin Day of incubation	Chick embryo			Eye balls		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
5	15.19	9.73	5.46	—	—	—
6	15.08	11.48	3.60	8.02	3.51	4.51
7	16.02	12.01	4.01	12.31	5.71	6.60
8	16.74	12.64	4.10	13.52	7.11	6.41
9	19.16	12.53	6.63	15.81	9.52	6.29
10	23.12	18.22	4.90	13.53	10.91	2.62
11	23.72	21.96	1.76	12.21	9.04	3.17
12	29.52	25.41	4.11	16.81	8.80	8.01
13	21.11	16.06	5.05	12.30	5.40	6.90
14	21.92	16.20	5.72	15.81	5.93	9.88
15	22.15	12.22	9.93	15.62	5.83	9.79
16	21.31	13.12	8.19	14.01	5.31	8.70
17	18.35	12.72	5.63	10.40	4.20	6.20
18	15.92	9.30	6.62	15.42	3.30	12.12
19	16.18	10.82	5.36	15.84	4.65	11.19
20	16.43	13.85	2.58	16.12	4.18	11.94

TABLE 3. On the vitamin C content of allantoic fluid, amniotic fluid and egg yolk during the incubation (mg%)

Portion Day of incubation	Allantoic fluid			Amniotic fluid			Egg yolk		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
6	2.68	0.93	1.70	3.41	2.34	1.07	5.21	0.28	4.93
7	3.84	1.95	1.89	2.32	1.31	1.01	8.33	0.34	7.99
8	3.23	1.84	1.44	2.38	1.43	0.95	7.22	0.46	6.76
9	3.15	1.68	1.47	3.19	2.71	0.48	5.73	0.56	5.17
10	4.75	1.75	3.00	3.01	2.52	0.49	5.87	0.52	5.35
11	6.52	2.05	4.47	1.92	1.30	0.62	4.10	0.64	3.46
12	7.21	2.32	4.89	2.11	1.31	0.80	4.70	0.60	4.10
13	6.84	2.53	4.31	1.65	0.78	0.87	6.25	0.63	5.62
14	5.83	1.71	4.12	2.30	1.42	0.83	5.83	0.68	5.15
15	5.21	2.20	3.01	1.92	0.88	1.04	5.60	0.84	4.76
16	6.90	1.07	5.83	1.50	0.82	0.68	4.96	0.76	4.20
17	5.90	0.57	5.33	1.18	0.55	0.63	3.78	0.65	3.13
18	5.24	0.53	4.71	1.43	0.51	0.92	4.71	0.59	4.12

CONCLUSIONS

1. The appearance of vitamin C in the chick embryo seems to be initiated simultaneously with the initiation of the incubation. The vitamin C content gradually increases. Moreover, in the case of embryo, the reduced vitamin C content is always larger than the other.

2. The vitamin C content in the eye balls fluctuates during the course of incubation. However, in the last half of the incubation, the oxidized vitamin C content is larger than the other.

3. In the case of allantoic fluid, the vitamin C content is inclined to a gradual increase. As soon as the vitamin C content began to increase, the oxidized vitamin C content becomes larger than the other.

4. In the case of amniotic fluid, the vitamin C content does not show any marked fluctuation during the whole course of incubation.

5. The total vitamin C content in the egg yolk is relatively larger in the first half of the incubation, but it gradually decreases in the last half of the incubation. Furthermore, most part of the vitamin C is oxidized type.

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