CHEMISTRY OF THE CHICK EMBRYO

V. THE ACCUMULATION OF CYTOCHROME OXIDASE*

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The important position occupied by cytochrome oxidase in the present day concepts of oxidative metabolism led us to study its accumulation during the development of the chick embryo. The data of Albaum and coworkers (1, 2) describe the increase in cytochrome oxidase only in terms of total amount. No relevant data on weight or other quantities are given with which the amount of the oxidase could be related to the size of the actively growing organism at the time. The changes in metabolism and development proceed in regular sequence and adequate description of the whole sequence of development requires that correlation be carried as far as possible. This has been the general aim of this series of papers and the present report presents the results of the determinations of cytochrome oxidase on a large number of embryos whose ages range from 3 to 16 days of incubation.

Test System—Cytochrome oxidase activity has usually been estimated by measuring oxygen consumption in a system in which reduced cytochrome *c* is supplied in excess by the addition of a reducing substance such as *p*-phenylenediamine, hydroquinone, or ascorbic acid. The test system adopted for this study was the following mixture: 0.4 ml. of cytochrome *c* (0.6 mg.), 2.2×10^{-5} mole; 0.4 ml. of 2 per cent *p*-phenylenediamine; 1.0 ml. of a suspension of material to be tested; 0.2 ml. of M phosphate buffer, pH 7.3. The reaction was carried out at 37.2° in Barcroft-Warburg respirometers. The *p*-phenylenediamine was tipped in from the side arm after temperature equilibration. Oxygen consumption was measured for 1 hour. The cytochrome *c* was sufficient to insure maximal oxidative activity (10). With each determination a control vessel was also included which contained no added cytochrome *c*. The oxygen consumption of this control vessel was subtracted from that of the vessel containing cytochrome *c* and the difference was used as the measure of cytochrome oxidase activity.

The oxygen consumption in the control vessel is the sum of several factors: (1) basal respiration of the tissue, (2) autoxidation of *p*-phenylenediamine, (3) non-cytochrome oxidase catalysis which is due to cytochrome *b*

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according to Stotz *et al.* (10), (4) oxygen consumption due to cytochrome *c* already present in the tissue. If the fourth of these factors is appreciable, then this vessel does not constitute a valid control for the estimation of oxidase activity. From the following considerations, it was concluded that the cytochrome *c* already present makes no significant contribution to the oxygen consumption in the control vessel.

1. Cytochrome *c* is very soluble and should be distributed throughout the aqueous phase of the system. If any were left in intact cells in a local high concentration or attached to "macromolecules" (9) in a particularly favorable position to react with the oxidase, then further grinding resulting in further dispersion of cytochrome *c* so attached should be reflected in decreased oxygen consumption in the control vessel. This effect was not observed even after long grinding in the homogenizers.

2. If we then assume that the cytochrome *c* is uniformly distributed and still contributes to the oxygen of the control, the addition of amounts of cytochrome *c* equal to that in the tissue should result in a measurable increase of oxidation. From the figures of Potter and DuBois (7) we find that the 10 day embryo contains 3.0 γ per gm. The addition of 0.3 γ of cytochrome *c* to a control vessel containing 100 mg. of tissue from a 10 day embryo had no effect on the oxygen uptake.

3. Potter and DuBois (7) reported at least a 6-fold increase in cytochrome *c* content from 6 to 12 days. During this time, it will be seen that a rise of only 50 per cent occurred in the control oxidation.

4. With concentrations of KCN reported by Stotz (10) to inhibit oxidase activity nearly completely, it was found that the control oxidation is inhibited only 25 per cent, while that of the vessels containing optimal amounts of cytochrome *c* is inhibited about 80 per cent. The more resistant system of the control vessels thus exhibits the same cyanide insensitivity as that reported for cytochrome *b*.

Embryological Material—Fertile eggs (from New Hampshire hens mated with Plymouth Rock cocks) collected in trap nests and received within 48 hours of laying were incubated at a temperature of 38.5° and at a relative humidity of 60 to 70 per cent. The eggs were rotated twice a day and removed in groups of three for analysis.

Preparation of Tissues—After removal from the incubator the embryos were freed of extraembryonic membranes, dried superficially, and weighed. They were then ground with 9 volumes of 1 per cent saline in glass homogenizers (8). Large embryos were minced in 9 volumes of saline in the Waring blendor for 20 seconds before being ground in the homogenizers. Long treatment in the blendor was found to inactivate the enzyme. 1 cc. portions (100 mg. of tissue) of these homogenates were pipetted into the Warburg vessels for the estimation of oxidase activity.

Results

Table I shows the effect of varying the amount of tissue in the test system used for this study. It demonstrates the strict proportionality of the amount of tissue to the oxidase activity. The lack of proportionality between the oxygen consumption of the control vessels and the amount of tissue in each one reflects the varied natures of the factors contributing to the oxidation in these vessels.

TABLE I

Oxygen Consumption by 10 Day Embryo Tissue Plus 8 Mg. of p-Phenylenediamine

Tissue, *mg.*	50		100		150	
Cytochrome *c*, $M \times 10^5$	0	2.2	0	2.2	0	2.2
	c.mm.	*c.mm.*	*c.mm.*	*c mm.*	*c.mm.*	*c.mm.*
O_2 used per hr.	30	132	51	258	69	370
Net O_2 per 100 mg. tissue		204		207		200

TABLE II

Accumulation of Cytochrome Oxidase in Chick Embryos

No. of embryos grouped	Average wet weight	Age calculated	Control, c.mm. O_2 per 100 mg. per hr.	Oxidase activity, c.mm. O_2 per 100 mg. per hr.
	mg.	*days*		
11	12.1	2.93	38	127
20	73.1	4.00	40	174
9	226	5.70	38	170
8	529	6.30	39	167
8	890	7.26	45	168
6	1,110	7.70	46	167
5	1,305	8.02	49	169
13	1,813	8.75	52	193
8	2,854	9.64	50	225
13	4,267	10.9	52	273
12	7,035	12.0	62	284
12	7,814	12.7	62	316
10	10,560	13.9	67	324
10	13,680	14.8	66	334
11	16,230	16.0	68	345

A total of 157 embryos was used for this study. Individual determinations were made on each except for very young embryos, in which case two or more were pooled to provide enough tissue for a determination (2 × 100 mg.).

The whole series was arranged according to weight and divided into con-

venient groups according to size. The average age of the group was calculated from the average weight as recommended by Levy and Palmer (4). These data are included in Table II which shows the average oxidase activities as well as the control values for each group.

DISCUSSION

Various workers have determined the dry weight on chick embryos at various stages of development (6). Different breeds of chicks have shown

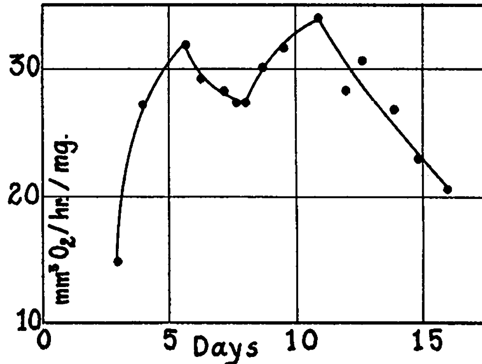

FIG. 1. Cytochrome oxidase activity per unit of dry weight against the calculated age in days.

TABLE III

Parameters of Phase Equations for Cytochrome Oxidase Accumulation

Log c.mm. of O_2 per mg. per hour = $i_Q + a_Q \log A$.

Interphase times, *days*	(3.0)*	4.3	7.9	11.8	(16.0)*

i_Q	−3.5†	−1.8	−3.3	−2.0
a_Q	6.3†	3.5	5.1	3.9

* The figures in parentheses indicate the beginning and end of the period of experimental observations.

† The figures for this phase are based on two points only.

only minor differences in these values for a given age. Since Albaum and Worley (2) have indicated that the cytochrome oxidase activity of chick embryo tissue is proportional to the dry weight, it seemed of interest to plot the oxidase activity on a dry weight basis against time. Fig. 1 reveals that the oxidase activity is not proportional to the dry weight. The dry weight figures used in these calculations are those of Murray (5).

The description of growth data by appropriate equations relating the quantity measured, Q, to time, A, is a useful device. The equation which we have used to describe the accumulation of material during growth is log

$Q = i_Q + a_Q \log A$ (3). It was noted that at various times (called interphase times) it is necessary to make abrupt adjustment of the parameters i_Q and a_Q in order to fit the data. This is usual in all forms of growth equations covering development. A period in which the parameters remain unchanged is called a phase of growth in our scheme. A satisfactory modification of the equation is obtained by combining equations for the substance of interest with those for weight (W) (4). By plotting logarithms of concentration (Q/W) against logarithms of age, a relative accumulation diagram results, the slopes and intercepts of whose phases are simply related to i_Q and a_Q of the accumulation equations (4).

When the data of Table II are plotted in the way described above, three phases of accumulation are evident, with a fourth indicated by a single point. The interphase times and phase accumulation parameters are shown in Table III as obtained graphically. Each phase except the earliest is determined by at least four points falling on a straight line in the plot of the log concentration (c.mm. of O_2 per mg. per hour) of cytochrome oxidase against the log age calculated (in days). The interphase time at 4.3 days has appeared in all the entities measured so far in the present series; namely, wet weight, nitrogen, dipeptidase, and aminopeptidase (4). The interphase at 11.8 days is present in nitrogen (11.5 days) and aminopeptidase (11.9 days) accumulation diagrams. The significance of these times in terms of the metabolic and physiological activities of the embryo is not evident. The phase constants and interphase times are to be regarded at present as descriptive devices. The rapid rise in oxidase activity beginning at the 8th day was noted by Albaum and Worley (2).

SUMMARY

1. The cytochrome oxidase activity in chick embryos between the incubation ages of 3 and 16 days has been measured.

2. The data are used to construct accumulation diagrams and phases of constant multiplication rates in logarithmic time are demonstrated.

3. The cytochrome oxidase activity is not proportional to the dry weight.

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