Biochemical Studies of the Developing Avian Embryo

IV. SOME RESPIRATORY PIGMENTS*

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In previous papers of this series we have demonstrated that cell-free preparations obtained from chick embryos are capable of catalyzing the individual reactions of the citric acid cycle as well as a number of typical reactions of terminal electron transport (1, 2). It has been shown that the activities of these enzymes vary as a function of embryonic development. Other workers have employed different methods to show that the tissues of the chick embryo can carry out the reactions of respiratory metabolism (3–7).

Yet Potter and Dubois (8) were unable to detect cytochrome c before the sixth day of development and Yaoi (9) maintained that the embryo contained no cytochrome c before the fourth day of development. A lack of this cytochrome would appear to be totally incompatible with the observations cited above. Carey and Grevelle (7), on the other hand, were able to demonstrate the presence of cytochromes a, b, c, and c1 in tissues of the 5-day-old chick embryo. In the present study spectrophotometric methods have been used to demonstrate the presence and some of the metabolic reactions of the various cytochromes in particles isolated from 4-day-old, and from the hearts and livers of 14-day-old chick embryos.

Coenzyme Q, a benzoquinone derivative, has been shown to be intimately associated with the processes of mitochondrial electron transport in adult vertebrates (10–19). We have been able to demonstrate the presence of this quinone in a number of tissues of the chick embryo, and to obtain some preliminary evidence for its functional role.

EXPERIMENTAL PROCEDURE

Tissue Preparations—Unincubated embryonated eggs from New Hampshire Red × Rock Cornish chickens were obtained from Indiana Farm Co-operative, Indianapolis. The eggs were incubated and treated as previously described (1). The tissue was excised, and classified according to the method of Hamburger and Hamilton (20).

Cell Fractionation—The fractionation technique used in our earlier work (1) could not be applied here since polyvinylpyrrolidone interfered with the spectrophotometry and some of the extraction procedures. The method used in this investigation was a modification of that described by Hogeboom (21): mitochondria were sedimented at 11,500 × g for 20 minutes and after washing resedimented at 21,000 × g for 60 minutes.

Methods for Obtaining Cytochrome Difference Spectra—Freshly isolated mitochondria were made up to a volume of 9.5 ml with 0.25 M sucrose, 0.1 M tris(hydroxymethyl)aminoethane buffer, pH 7.4. Two reaction mixtures were prepared in glass-stoppered test tubes: the sample contained 3.0 ml of the mitochondrial preparation, 0.02 ml of sodium azide (90 mM), and 0.1 ml of ADP (3 mM). The blank contained 3.0 ml of the mitochondrial preparation, 0.02 ml of Amytal (0.062 g per ml in 95% ethanol), and 0.38 ml of water. The sample was flushed with nitrogen, and oxygen was bubbled into the blank. Both reaction mixtures were incubated for 30 minutes at 37°. At the end of the incubation period 0.3 ml of sodium deoxycholate (2 g per ml) was added to each mixture. The spectrum of the sample versus the blank was then rapidly taken by means of a Cary model 11 recording spectrophotometer with a slide-wire giving full scale deflection for an A = 0.100.

Extraction and Determination of Mitochondrial Coenzyme Q—Freshly isolated mitochondria were resuspended in 0.25 M sucrose buffered at pH 7.4 as before, to yield a final concentration of 30 to 50 mg of protein (biuret) per ml. Two methods for the extraction of coenzyme Q-like components were employed.

(a) Acetone extraction: the direct procedure developed by Lester and Fleischer (17) for the isolation of coenzyme Q from adult heart mitochondria was used. The acetone extract was evaporated to dryness on a rotary flash evaporator, and the yellow residue dissolved in 1 to 2 ml of 95% ethanol. Spectra were taken in this solvent by means of the Cary model 11 recording spectrophotometer. The reduced spectra were obtained by the addition of a small volume of concentrated aqueous KBH4 directly to the cuvette.

(b) Cyclohexane extraction: the techniques described by Green et al. (19)† for the isolation and determination of Qb in adult heart mitochondria was used. The tissue was incubated at 37° for 30 minutes, and the mitochondrial suspension was centrifuged at 21,000 × g for 60 minutes. The resulting supernatant was extracted with cyclohexane, and the resulting yellow residue dissolved in 1 to 2 ml of 95% ethanol. Spectra were taken in this solvent by means of the Cary model 11 recording spectrophotometer. The reduced spectra were obtained by the addition of a small volume of concentrated aqueous KBH4 directly to the cuvette.

Coenzyme Q was identified by comparing the spectra obtained with those of authentic coenzyme Q, and by comparing the absorption maxima with those reported in the literature. The method of analysis was a modification of that described by Lester and Fleischer (17): coenzyme Q in the sample was reduced with potassium bichromate, and the reduced coenzyme Q was oxidized with potassium bichromate. The difference spectrum was taken and the absorption maxima were compared with those of authentic coenzyme Q.

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1 And private communication.
2 Qb is an abbreviation for

\[
\text{CH}_3\text{O} - \text{CH}_2 - \text{CH} = \text{C} - \text{CH}_3 + \text{H} \rightarrow \text{CH}_3\text{O} - \text{CH}_2 - \text{CH} = \text{C} - \text{CH}_3 \text{H} (12, 13, 22).
\]

This compound is identical with that referred to as ubiquinone-(50) by Morton et al. (23).
its oxidized and reduced forms, from aqueous particle suspensions was adapted for use with preparations of embryonic origin; the particles were treated with HClO₄ (final concentration 0.2 M) and extracted with cyclohexane (5 ml per each 2 ml of suspension). The ternary mixture was centrifuged, the cyclohexane layer removed and evaporated to dryness as before, the residue taken up in 95% ethanol and the spectra examined. Reduction with KBH₄ as before and determination of the ΔA₅₃₅ (oxidized - reduced) permits the calculation of the concentration of oxidized coenzyme Q in the steady state; ΔA₅₃₅, i.e. the differential absorbancy of a solution containing 10 mg of Q₁₀ per ml is taken as 142 (12); assuming the molecular weight of Q₁₀ to be 849.3 this leads to a Δε₅₃₅, the differential millimolar extinction coefficient, for Q₁₀-like compounds of 12.08. For a determination of the total coenzyme Q in the preparation a second aliquot of the particle suspension was mixed with an equal volume of 0.1 M KH₂PO₄ and the mixture heated in a boiling water bath for 15 minutes. This procedure converts coenzyme Q entirely to the oxidized form. The cooled suspension was then treated with cyclohexane, and the rest of the procedure followed as above.

Paper Chromatography—Fifty microliters of authentic Q₁₀, vitamin K₁, and d-α-tocopherol, as well as 50 to 100 μl of the cyclohexane or acetone extracts, prepared as described above, were spotted on sheets of Whatman 3 MM chromatographic paper, previously impregnated with Dow Silicone Fluid 550. The chromatograms were then developed by the ascending technique using the n-propanol-H₂O systems (4:1 and 7:3) described by Lester and Ramasarma (22).

After development the chromatograms were dried and various compounds identified and characterized by the following methods: (a) reaction with KMnO₄ (22); (b) reduction of neotetrazolium chloride either with (oxidized form) (22), or without (reduced form) prior reduction with KBH₄; (c) the sensitive quinone sulfate spray for phosphate derivatives, described by Rorem (24); and (d) a visual observation for yellow color.

Spectra—All spectra were taken with the Cary model 11 spectrophotometer, with the use of cells with a path length of 1.00 cm, and either a 0–0.100 or a 0–2.50 absorbancy slide-wire. The spectra shown in the various figures are tracings of the original recordings.

Materials—DPNH and cytochrome c were obtained from the Sigma Chemical Company. The cytochrome was reduced, when required, by the method of Smith (25). Amytal was a gift of Dr. O. K. Behrens, Eli Lilly and Company. Antimycin A was purchased from the Wisconsin Alumni Research Foundation, Madison, Wisconsin. We are indebted to Professors David E. Green and F. Crane, as well as to Dr. Karl Folkers of Merck Sharp and Dohme for the samples of coenzyme Q (Q₁₀) used as standards in this investigation. The other standards used were Vitamin K₁, Vitamin E (d-α-tocopherol), and Vitamin A (acetate), purchased from Nutritional Biochemicals; all appeared to be chromatographically pure in the two solvent systems used. All solvents were spectral quality grade, and the water was doubly glass distilled.

RESULTS

Cytochrome Difference Spectra

Fig. 1 shows the difference spectrum obtained when heart mitochondria of 14-day-old chick embryos were reduced with succinate. Absorption bands due to cytochromes a, a₃, b, and...
c³ in addition to that of flavoprotein may be seen. A rough approximation of the relative content with regard to the various cytochromes present may be made by the method of Chance and Williams (26) as modified by Raw and Mahler (27). This involves the use of extinction coefficients determined with optically pure preparations of individual cytochromes, and the use of wave lengths where interference between them is at a minimum.

The values used for spectra and extinction coefficients were: for cytochromes a and a₃, the spectra of Smith (28) and extinction coefficients of Keilin and Hartree (29); for cytochrome b, spectrum and extinction coefficients of Hubscher et al. (30); for cytochrome c, the spectra of Margoliash (31) and the extinction coefficients of Chance and Williams (32). The following equations were used to calculate the approximate concentrations of the various cytochromes in the preparations:

\[ \Delta O.D. 445 - \Delta O.D. 455 = 43.8 (a₃) \]
\[ \Delta O.D. 605 - \Delta O.D. 630 = 9.1 (a) \]
\[ \Delta O.D. 564 - \Delta O.D. 575 = 15.2 (b) \]
\[ \Delta O.D. 550 - \Delta O.D. 540 = 19.0 (c) \]

The values in brackets refer to the millimolar concentrations of the respective cytochromes. With these equations and the spectrum of Fig. 1 it can be seen that the approximate molar ratios of the cytochromes were close to unity.

\[ a₃:a:b:c = 1.0:1.4:1.1:1.0 \]

Fig. 2 shows the difference spectrum obtained with mitochondria from the livers of 14-day-old chick embryos. Absorption bands due to cytochromes a, a₃, b, c, and flavoprotein may be seen. Approximate values for the relative cytochrome content, calculated as described above, were as follows:

\[ a₃:a:b:c = 1.5:1.1:1:1.1:0 \]

Once again the values were close to unity.

* Cytochromes c and c₁.

Fig. 3 shows the difference spectrum observed with mitochondria obtained from 4-day-old chick embryos. Since it was difficult to obtain this tissue in large amounts the spectrophotometric analysis was carried out on a dilute homogenate. In this experiment the error due to light scattering was large enough to interfere with the quantitative determinations of the cytochromes. Qualitatively, cytochrome a, a₃, b, and c were seen to be present in this tissue.

Fig. 4 shows the same preparation reduced with sodium dithionite. Since no new peaks emerged on complete reduction it can be inferred that the cytochromes present were all functional.

Microsomes were prepared from the livers of 14-day-old chick embryos and were washed two times with 0.25 M sucrose. A difference spectrum was obtained with this preparation using sodium dithionite as the reducing agent and is shown in Fig. 5. The spectrum appears to be mainly that of cytochrome b₃ (33, 34). Slight amounts of contaminating pigment may be attributed to the presence of disintegrated mitochondrial fragments in the microsomal preparation.⁴

Antimycin A was found by Ahmad et al. (35) to have a powerful inhibitory effect on cellular respiration. Potter and Reif (36) subsequently showed that it inhibited DPNH oxidase. An inhibition of embryonic DPNH oxidase by the antibiotic was reported in a previous paper of this series (2). Duffey and Ebert (37) have reported that the antibiotic inhibits the differentiation of heart tissue in the early chick embryo. In adult tissue antimycin A is believed to act at a site between cytochrome b and cytochrome a₁ (32, 38, 39).

Fig. 6 shows the difference spectrum of antimycin A-inhibited heart mitochondria obtained from 14-day-old embryos. The solid trace, which represents the spectrum taken immediately, shows the reduction of cytochrome b with some reduction of the

⁴ Mitochondria obtained from embryonic preparations appear to be more fragile than their adult counterparts. Experimental data related to this will be presented in a future publication.
FIG. 3. The cytochromes of mitochondria from 4-day embryos. Difference spectrum obtained with mitochondria isolated from a homogenate of whole 4-day-old chick embryos. Method described in the text. Protein content: 9.2 mg X ml⁻¹.

(a + a₃) a
(b + c)α
(c + c₃)α

Δ O.D.
.01

FIG. 4. The hemoproteins of mitochondria from 4-day embryos. Spectrum of mitochondria isolated from a homogenate of whole 4-day-old chick embryos and completely reduced with dithionite. This is to be contrasted with Fig. 3 where the reduction of the (a + a₃) and c components, possibly due to the intervention of an antimycin A-insensitive pathway.

After standing for 2 hours or more at room temperature the nature of the spectrum changed drastically. The dotted trace shows the spectrum after the preparations had stood for 6 hours. The extent of reduction of cytochrome b had decreased considerably whereas that of (c + c₃) and (a + a₃) had increased perceptibly.

Fig. 7 shows that a similar situation prevailed when mitochondria from livers of 14-day-old embryos were enzymatically reduced in the presence of antimycin A.

There are a number of possible explanations for the phenomena observed. It is possible, for instance, that the deoxycholate liberated from the preparation a component capable of binding antimycin A more strongly than the inhibitable respiratory factor. The reversal of inhibition would then be similar to the
The hemoproteins of embryonic microsomes. Spectrum of microsomes isolated from the livers of 14-day-old chick embryos and completely reduced with dithionite; protein content 10 mg X ml⁻¹.

Fig. 6. The effect of antimycin A on the cytochromes of embryonic heart mitochondria. Difference spectrum obtained with mitochondria isolated from the hearts of 14-day-old chick embryos. The blank (oxidized) was made up as described in the text whereas the experimental sample contained 50 pg of antimycin A (added as 0.1 ml of a 0.05% solution in 95% ethanol) instead of azide. Both samples were incubated at 37° for 40 minutes and were continuously saturated with O₂ during this incubation period. At its end deoxycholate was added and the spectra taken immediately, (---); after 6 hours (----). Protein content: 13.1 mg X ml⁻¹.

albumin reversal found by Reif and Potter (39). Alternatively the deoxycholate may have acted by liberating mitochondrial cytochrome c₁, causing electron transport to by-pass the inhibitable factor between cytochromes b and c₁. The reaction could then proceed by means of a direct cytochrome c reductase and cytochrome c oxidase pathway. This would be somewhat analogous to the "opening" phenomenon described by Green et al. (40). Finally the cytochrome b may have been "squeezed out" of the sequential chain of cytochromes, and the reaction may have assumed a pattern similar to that observed by Chance (41) in a nonphosphorylating or otherwise partially degraded preparation.

In any event the extensive reduction of cytochrome b observed initially is a strong indication that the particles used during this investigation were intact phosphorylating mitochondria rather than some degraded type of electron transport particle.
FIG. 7. The effect of antimycin A on the cytochromes of embryonic liver mitochondria. Difference spectrum obtained with mitochondria isolated from the livers of 14-day-old chick embryos. Protocol similar to that of Figure 6; protein content: 17.8 mg X ml⁻¹.

FIG. 8. Spectra of a cyclohexane extract of embryonic mitochondria. Mitochondria (protein content 564 mg) were isolated from the livers of 316 13-day-old chick embryos, deproteinized with HClO₄, and extracted with cyclohexane. After evaporation to dryness the residue was taken up in 2.5 ml of 95% ethanol. For the curve marked S the spectrum of 0.3 ml of this solution diluted to 0.9 ml with ethanol was taken directly. The spectrum marked Red is that of the same solution reduced with KBH₄. For the spectrum marked Ox another aliquot of the original solution (0.8 ml diluted to 1.8 ml with ethanol) was shaken with Ag₂O, the latter removed by centrifugation, and the spectrum taken.

Occurrence of Coenzyme Q

Spectrophotometric Evidence—When mitochondria isolated from the hearts or livers of 13- or 14-day-old chick embryos were extracted by any of the methods devised for the isolation of coenzyme Q, as described above, and their spectra examined, curves similar to that shown in Fig. 8 were obtained. Here S represents the spectrum of the mixture as isolated, Red corresponds to that of the same sample after reduction by KBH₄, and Ox is that of a separate sample but of the same preparation, oxidized by Ag₂O. Two conclusions may immediately be drawn from experiments of this kind: (a) evidently two (or more) components contribute to the spectra—one, reducible by KBH₄,
Fig. 9. Difference spectrum of a cyclohexane extract of embryonic liver mitochondria. Similar to the experiment of Fig. 8 except that 311 embryos were used yielding 734 mg of mitochondrial protein. The residue obtained from the cyclohexane extraction was taken up in 2.5 ml of 95% ethanol and aliquots of 0.4 ml diluted to a final volume of 1.4 ml with ethanol were used for the difference spectrum \( \Delta \text{OD}_{\text{oxidized-reduced}} \). \( \text{OD}_{\text{oxidized}} \) is the absorbancy of the solution as isolated whereas \( \text{OD}_{\text{reduced}} \) is that of another sample reduced with KBH₄. The difference spectrum of a 1% solution of authentic Q₁₀ \( (\Delta \text{AA}_{\text{Q₁₀}}) \) treated similarly is shown for comparison.

with a broad maximum centered around 275 \( \mu \)m, presumably coenzyme Q, or a closely related entity, and one (or more) not reducible by KBH₄, responsible for the three-banded appearance of the spectrum, especially apparent after prior reduction of the first entity, with maxima at 270, 279 to 280, and 325 \( \mu \)m; (b) only a relatively small additional increment of compounds absorbing at 275 \( \mu \)m appears after oxidation by Ag₂O; i.e. as isolated, most of this material is already in the oxidized form. As a matter of fact many samples gave a spectrum indistinguishable from Q₁₀ even without prior oxidation by Ag₂O.

In order to identify the 275 \( \mu \)m component as a member of the coenzyme Q-family, several additional experiments were performed. When the difference spectrum (oxidized-KBH₄ reduced) of a typical embryonic preparation was taken and compared with that of an authentic sample of Q₁₀, the results shown in Fig. 9 were obtained. The close resemblance is apparent. In another experiment the mixture of components obtained after cyclohexane extraction of 13-day liver mitochondria was subjected to chromatography on an alumina column. After development of the chromatogram with 

\[
\text{CHCl}_3\text{-methanol, in proportions of 80:20, 60:40, and 40:60, respectively.}
\]

Three different solvents of increasing polarity were then used to elute the column (CHCl₃-methanol, in proportions of 80:20, 60:40, and 40:60, respectively). The first yellow band was eluted by the least polar solvent mixture and in this behaved similarly to an authentic sample of Q₁₀, the results obtained. The fast moving component was then evaporated to dryness, dissolved in ethanol, and its spectrum taken and compared to an authentic sample. The results of this experiment are shown in Fig. 10, Curve C and Q₁₀. Shown there also is the spectrum of an acetone extract of mitochondria isolated from a homogenate of whole 4-day embryos. Evidently a coenzyme Q-like entity was present in this extract and its spectrum was relatively uncomplicated by the presence of the additional components which make their appearance in extracts of mitochondria isolated from the tissues of older embryos.

Chromatographic Evidence—A typical chromatogram of a cyclohexane extract of liver mitochondria from 13-day embryos is presented in Fig. 11, and the results of several representative experiments are summarized in Table I.

As can be seen, there is evidence for the presence of the same two compounds, giving the characteristic color reactions of coenzyme Q (22), in all embryonic extracts examined. The slower moving component may be tentatively identified as Q₁₀. The fast moving, probably more highly polar component, appears to be present in 5- to 10-fold excess over the slower moving component as indicated by the relative intensities of their respective color reactions. Yet in spite of its high concentration, ubiquity and apparent similarity in color tests there are several reasons why we do not consider it to be similar to coenzyme Q in metabolic function and importance: (a) comparison of the intensity of the slower migrating spot with authentic samples of Q₁₀ indicated a concentration of the former of the same magnitude as that calculated from spectrophotometric data; for example in one experiment, that depicted in Fig. 8, the concentration of total coenzyme Q in the sample as determined spectrophotometrically was 1.64 \( \mu \)g per mg of protein; the concentration of Q₁₀ by paper chromatography was 1.6 \( \mu \)g per mg of protein. In four other experiments the spectrophotometrically determined coenzyme Q was greater than that estimated from papergrams by a factor of 1.3 ± 0.4. Thus all the reducible material responsible for the characteristic absorption spectrum with \( \lambda_{\text{max}} = 275 \mu \)m was accounted for by the Q₁₀-like component, and the rapidly migrating component could not be considered to be coenzyme Q-like in absorption spectrum. (b) The rapidly mi-
Fig. 10. The spectrum of coenzyme Q preparations from embryonic materials: From a cyclohexane extract of the mitochondria isolated from 934 4-day-old embryos (total mitochondrial protein 80 mg) after concentration and solution in 2.5 ml of 95% ethanol the spectrum marked 4-day was obtained. For the spectrum marked C the mitochondria were isolated from 629 livers of 13-day-old embryos. The particles were deproteinized with HClO₄, extracted with cyclohexane, the solvent removed under reduced pressure and the residue dissolved in 1 ml of CHCl₃, and chromatographed on a column (13 × 1.6 cm) of Merck acid-washed chromatography alumina (10 g). The chromatogram was developed with 30 ml of CHCl₃, and the most rapidly migrating yellow band eluted with 10 ml of a mixture containing 8 ml of CHCl₃ and 2 ml of absolute methanol. The solvents were removed in a flash evaporator, and the residue dissolved in ethanol and the spectrum taken.

Grating component was not reduced by KBH₄ and was capable of interacting with neotetrazolium even without prior reduction; if at all similar to coenzyme Q it was therefore present as the hydroquinone. Yet unlike the hydroquinone of coenzyme Q it did not appear to be oxidizable by Ag₂O, and the Q₁₀ isolated under identical conditions (i.e. the slower component) existed almost entirely as the quinone (cf. preceding section). (c) In a metabolic experiment (see below) in which the oxidation state of coenzyme Q could be shown to be profoundly affected, the unknown rapidly moving component appeared to remain entirely in the reduced state. We have therefore not so far pursued its identification. This, the elucidation of its structure and its relationships, if any, to some of the lipid constituents of chick liver described by Cunningham and Morton (42) and to the other light-absorbing entities in our extracts, will form the subject of a subsequent communication.

Metabolic Significance—With the tentative identification of Q₁₀ as a component of embryonic mitochondria two questions remained to be settled: was it involved in the metabolic function of the particles in a manner analogous to their adult counterparts, and what were its quantitative relationships to other members of the electron transport sequence? In order to obtain some indication concerning the first point we carried out an experiment the results of which are summarized in Fig. 12. Here we incubated mitochondria isolated from the hearts of 14-day-old embryos under various conditions and isolated the coenzyme Q fraction after HClO₄ deproteinization and cyclohexane extraction (19). Curve I was obtained from mitochondria incubated for 30 minutes at 37° with no additions and thus corresponded to the steady state in the presence of endogenous substrate; it will be seen that coenzyme Q was almost entirely in the oxidized state (see also Fig. 8). For Curve II, succinate, ADP, and azide were added, and now, in the presence of excess substrate with the terminal oxidase system blocked, Q became fully reduced. Several features emerged from these two experiments: (a) the intramitochondrial coenzyme Q of embryonic particles like its adult counterpart was capable of being reduced by succinate and oxidized through the cytochrome oxidase system; (b) if reduced through metabolic action the Q-hydroquinone was stable to the isolation procedures used and could be estimated as such; (c) therefore under ordinary conditions, the intramitochondrial Q in the steady state was almost entirely in the oxidized form. In the experiment of Curve III Amytal but no substrate was added. Since the extent of reduction here was greater than in Curve I we may conclude that in this system, at least, Amytal did not appear to block the reduction of Q, i.e. that the bulk of the endogenous reducing equivalents affecting this component were probably not provided by substrates reducing intramitochondrial DPN (26). It is also possible that the oxidation of reduced Q may have been somewhat Amytal-sensitive. In the last experiment, that of Curve IV, antimycin A but no substrate was added. A very considerable fraction of the total reducible coenzyme Q actually became reduced under these conditions. These observations permit us to assign a role to intramitochon-
Table I

Paper chromatographic data for extracts of embryonic mitochondria

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Age of embryo</th>
<th>Source of mitochondria</th>
<th>Extraction method</th>
<th>Solvent system</th>
<th>Compound</th>
<th>RF</th>
<th>Method of detection*</th>
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<tbody>
<tr>
<td>I</td>
<td>13 days</td>
<td>Liver</td>
<td>Acetone</td>
<td>4:1</td>
<td>K₁</td>
<td>0.63</td>
<td>+</td>
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<td>A</td>
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<td>13 days</td>
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<td>Q₁₀</td>
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<td>0.99</td>
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<td>V</td>
<td>13 days</td>
<td>Heart (a) HClO₄-cyclohexane (b) K₂HPO₄-heat-cyclohexane</td>
<td></td>
<td>4:1</td>
<td>Q₁₀</td>
<td>0.43</td>
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<tr>
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<td>Liver</td>
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<td>4:1</td>
<td>Q₁₀</td>
<td>0.42</td>
<td>+</td>
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* Detection methods (see text): a: KMnO₄; b: KBH₄-neotetrazolium; c: fluorescence after quinine sulfate; d: yellow color; a plus (+) indicates positive result; a minus (−) a negative result; a blank indicates not examined.

drial coenzyme Q and to cytochrome b (see Fig. 7, obtained under identical conditions) as two of the components of an antimycin-sensitive pathway of electron transport in embryonic tissues. The block in this pathway must then occur during the oxidation of reduced Q by the azide-sensitive oxidase, and before the reduction of cytochrome c₁ (cf. Fig. 7). That the oxidation of reduced coenzyme Q is antimycin-sensitive confirms the findings of Humphrey and Redfearn (18) and of Green et al. (19) with particles isolated from adult mammalian hearts.

Quantitatively, in four different experiments the coenzyme Q content of 13- or 14-day liver mitochondria extracted with HClO₄-cyclohexane was found to be 0.54, 0.62 (experiment of
Fig. 12), 0.84, and 1.64 μg per mg of protein. This then corresponded to a 1.1 × 10^{-4} mmole of this component per mg of protein compared to 3.9 × 10^{-4} mmole of cytochrome c per mg of protein (see Fig. 2). In liver mitochondria from 13-day-old embryos we therefore found a Q-cytochrome c ratio of 2.8. In mitochondria from 14-day hearts the coenzyme Q concentration was estimated to be 3.9 μg per mg of protein or 4.6 × 10^{-7} mmole per mg of protein. Their cytochrome c concentration (Fig. 1) was 3.1 × 10^{-5} mg per mg of protein. Thus these particles showed a Q-cytochrome c ratio of 15. Mitochondria isolated from homogenates of whole 4-day embryos contained 0.23 μg or 2.8 × 10^{-7} mmole of coenzyme Q per mg of protein.

The coenzyme Q content of mitochondria isolated from embryonic organs thus compares favorably with that reported for those of adult origin by Lester and Crane (13). Their data also suggest that mitochondria isolated from adult mammalian heart contain a higher concentration of coenzyme Q than do the corresponding particles of liver. This generalization appears to be equally valid for embryonic material.

**DISCUSSION**

Recent interest in the pathways of terminal respiration of rapidly proliferating tissues has been stimulated by the hypothesis of Warburg that in malignancy the controlling biochemical lesion leads to an irreversible damage in the respiratory mechanism and to an increase in glycolysis (43). Indeed, evidence was adduced that malignant tissue contains an unusually low concentration of cytochrome c and an abnormally low cytochrome oxidase activity (44). Furthermore the concentration of cytochrome c in malignant rat tissue appeared to be inversely related to tumor size (45).

By the use of refined spectrophotometric techniques Chance and Hess have been able to demonstrate that ascites tumor cells at least showed no impairment in their capacity to carry out the reactions of terminal electron transport (46–50). In mitochondria isolated from these cells there is no deficiency of cytochrome c and no apparent damage to the other respiratory carriers; the respiratory chain is fully capable of bringing about electron transport and oxidative phosphorylation. Any decrease of respiration relative to glycolysis observed with the intact cell can not therefore be due to any intrinsic lack in, or modification of, the respiratory chain or any of its components. Rather, to quote these investigators “it is concluded that respiratory metabolism of the intact cell is limited by metabolic control.”

Embryonic and malignant tissues share the property of rapid proliferation, controlled and purposeful in the former, uncontrolled in the latter. Embryonic tissue too was reported to exhibit an abnormally low cytochrome content (8, 9) and an active glycolysis (51). Among others, it was considerations such as these which prompted us to inquire whether changes or impairment in the respiratory mechanism might be a property of this rapidly proliferating system and bear some relationships to embryonic development and differentiation. In our studies so far we have been able to demonstrate that embryonic tissues of the chick, even those of 4-day embryos, have the capacity of catalyzing all the reactions of terminal respiration, including those of the citric acid cycle and of the electron transport chain (1, 2). This report indicates that like those of ascites tumor cells, the mitochondria of embryonic tissues down at least to the fourth day of development are analogous qualitatively and quantitatively to those of normal, adult, vertebrate tissue. There is no deficiency of any of the normal cytochrome components including cytochrome c and they interact normally in the orderly metabolic sequence established previously for a variety of systems. Coenzyme Q is found in significant concentrations in the mitochondria of all embryonic materials tested and apparently has the same structure (i.e. Q10) and potential role there as it does in the adult chick. Some of the quantitative relationships are summarized in Table II. Given therefore this equal capacity of adult and embryonic respiratory particles, any metabolic differences between them must be due to either a difference in subcellular organization or control. We will attempt to deal with these aspects of the problem in future communications.

**SUMMARY**

1. Mitochondria isolated from tissues of the developing chick embryo have been shown to contain a qualitatively normal complement of cytochromes as early as the fourth day of development. Cytochromes a, a', b, and (c + c') have been identified by spectrophotometric techniques.

2. These cytochromes appear to fulfill their normal metabolic roles since they are all reducible by succinate (in the presence of azide to block cytochrome oxidase), and since antimycin exerts its block at the locus previously established in adult systems, i.e. between cytochromes b and (c + c').

3. Cytochrome b1 is the preponderant hemoprotein component of embryonic microsomes as it is in the adult.
Electron transport particle embryonic tissues is compared and discussed in relation to similar observations of Chance and Hess with mitochondria from ascites damage in the terminal respiratory sequence and capacity of previously reported for actively respiring adult tissues.

Relative proportion to members of the respiratory chain appear entirely analogous to that previously reported for respiratory here as with cow heart, i.e., a factor of 40.

Mitochondria from active respiring adult tissues from adult organisms.

Mitochondria from 1P day chick embryonic liver and 14-day hearts 10a.

Note Added in Proof—Laidman et al. (Biochem J., 74, 541 (1960)) have now obtained substance SC in pure form and shown it to be a chromenol isomer of \( Q_{10} \). Both the qualitative (\( \lambda_{\text{max}} = 275,283,332 \text{ nm} \)) and quantitative features of its spectrum bear a striking resemblance to that of our unknown component (cf. Fig. 8), which may therefore be either ubiquichromenol or, possibly, a derivative such as a phosphate ester (cf. Table I, Expt. III). The use of the extinction coefficient cited by Laidman et al. leads to an estimate of approximately \( 4 \times 10^{-6} \) mmole ubiquichromenol-like material per mg of mitochondrial protein in embryonic liver and of \( 10^{-5} \) mmole per mg protein in embryonic heart mitochondria.
Biochemical Studies of the Developing Avian Embryo: IV. SOME RESPIRATORY PIGMENTS
Ludwig Brand, Carol Dahl and H. R. Mahler


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