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EXPLANATION OF PLATE 3

- (a) Ouchterlony plate in which an antiserum to the serum proteins of a tumour-bearing rat was placed in the centre well. The other wells contained either whole serum from normal rats or serum from tumour-bearing rats diluted with saline as indicated. Photographed after 12 days.
- (b) Ouchterlony plate set up exactly as described in (a) except that the agar contained 1% (v/v) of normal-rat serum.
- (c) Ouchterlony plate in which the centre well contained antiserum to tumour serum absorbed with one-fifth of its volume of normal-rat serum. The side wells contained either tumour serum or normal serum diluted with saline as indicated. Photographed after 10 days.

Mitochondria from Embryonic Tissues of the Chick

1. PREPARATION, CHARACTERIZATION AND SOME ENZYMIC PROPERTIES

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Whereas the isolation and properties of subcellular particles from normal adult tissues (review: Hogeboom & Schneider, 1955), tumour tissue (Schneider, 1946), plant tissues (review: Hackett, 1955) and yeast (Linnane & Still, 1955) have been extensively studied, no comparable work seems to have been done with embryonic tissues. Claude (1938, 1940) centrifuged particles from a homogenate of previously frozen chick-embryo pulp in phosphate buffer; they contained some ribonucleic but no deoxyribonucleic acid. Steinbach & Moog (1945) and Moog & Steinbach (1946) obtained granules from homogenates of chick embryos in water by centrifuging at room temperature, and studied their phosphatase and adenosine triphosphatase activities.

Recknagel (1950) homogenized eggs of the frog *Rana pipiens* in phosphate buffer and separated a granular fraction which contained about 70% of the cytochrome oxidase activity of the homogenate, with a much higher specific activity, and which stained with Janus green B. A similar mitochondrial fraction, in which the cytochrome and succinate oxidases were localized, was obtained by

Boell & Weber (1955) from homogenates in sucrose solution of embryos of the toad *Xenopus laevis*.

Nuclear, mitochondrial and microsomal fractions have been obtained by differential centrifuging of homogenates in sodium chloride-phosphate medium of embryos of the grasshopper *Melanoplus differentialis* (Bodine & Lu, 1950, 1952); all the succinate oxidase activity was in the mitochondrial fraction.

Hutchens, Kopac & Krahl (1942) separated from homogenates of eggs of the sea urchin *Arbacia punctulata* a 'mitochondrial' fraction which, however, contained only 1% of the cytochrome oxidase activity. On the other hand, Weber is reported by Boell & Weber (1955) to have found that homogenates of eggs of *Paracentrotus lividus* yield large granules which sediment between 600 and 12 000 g, have the characteristics of mitochondria and contain 90% of the cytochrome oxidase activity of the homogenate.

Clearly, cell-fractionation studies with embryonic tissue have so far been scanty and incomplete. Attempts have been made to separate a fraction containing enzymes that are associated with mitochondria in adult mammalian tissues, but in few cases have the workers carried out the fractionation in sucrose medium which, like certain special salt

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solutions (Chappell & Perry, 1954), allows the preparation of satisfactory particles. In particular, hypotonic and phosphate-containing media are likely to cause severe damage to the mitochondria (see, for example, Dituri, Shaw, Warms & Gurin, 1957).

The work described in this and the accompanying paper (Carey & Greville, 1959) represents an attempt to apply modern biochemical criteria to the problem of the nature and enzymic constitution of subcellular particles in embryonic tissue. Chick embryos were chosen, as they were available throughout the year. They were used whole after incubation for 5 days. In earlier stages, the amount obtainable would be very small, and in later stages, owing to considerable differentiation, the tissues might become more characteristic of the adult state.

EXPERIMENTAL

Abbreviations

ATP, Adenosine triphosphate; CoA, coenzyme A; DNA, deoxyribonucleic acid; DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetra-acetic acid; RNA, ribonucleic acid; tris, 2-amino-2-hydroxymethylpropane-1:3-diol.

Materials

Water distilled in glass was used and, when possible, AnalaR reagents. Acidic substances were neutralized with NaOH before addition to media.

The specimen of CoA, obtained some years previously from Pabst Laboratories, Milwaukee, Wis., U.S.A., was found by the method of Kaplan & Lipmann (1948) to have an activity of 208 units/mg. (approx. 50% purity).

Cytochrome *c*, prepared by the method of Keilin & Hartree (1945), was purified by adsorption on the ion-exchange resin IRC 50 (Margoliash, 1954).

Tris (L. Light and Co. Ltd.) was recrystallized from ethanol; 'Sigma 121' (tris supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A., for use as a primary volumetric standard) was used without further purification.

EDTA (L. Light and Co. Ltd.) was recrystallized from water.

Commercial DNP was recrystallized once from ethanol and twice from aq. ethanol; m.p. 112–113°.

The crystalline disodium salt of ATP was obtained from the Sigma Chemical Co., and glutathione from The Distillers Co. Ltd.

Preparative methods

Production of embryos. The eggs, all Rhode-Island Red × Light Sussex, were supplied by Withers and Son, Appelby Farm, Egerton, Ashford, Kent, who collected them within 4 hr. of laying. They were incubated at 38–39°, and the stage of development of embryos was determined by comparison with illustrations in Lillie's *The Embryology of the Chick* (Hamilton, 1952). Here, '5-day embryo' refers to embryos in stages 25–27, the majority being at stage 26. The stages of later embryos were not so accurately determined.

Separation of cell fractions. (a) Whole embryos: about eight embryos from approximately 12 eggs were collected in 0.31M-sucrose or 0.15M-KCl at room temperature and, after freeing from extra-embryonic membranes and yolk, transferred to ice-cold preparation medium (see below). These operations were completed in 5–8 min. Subsequent manipulations were carried out at 0–2°. The embryos (1.2–1.5 g.) were homogenized in 10 vol. of fresh medium by brief use (about 30 sec.), at low speed (300–500 rev./min.), of a Potter-Elvehjem device with Perspex rotor. Differential centrifuging was then carried out as shown in Table 1, which also gives the symbols assigned to the various fractions (cf. de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). When required for enzymic studies, fraction *M* was washed twice or thrice on the centrifuge, each time with a volume of medium equal to five to eight times the total volume of the embryos and centrifuging for the same time and with the same centrifugal force as in the original sedimentation of the mitochondria. (b) Liver tissue: this was treated similarly, except that material from hatched chicks was finely cut with scissors before homogenization.

Extracts from acetone-dried powders. Washed mitochondria from about 30 g. of liver of 12- to 14-day chicks, or from 6 to 8 g. of liver of 16-day embryos, were prepared with sucrose medium III (see below) and suspended in 5–10 ml. of the same medium at 0°. Acetone at –5° (20 vol.) was added, and the ppt. centrifuged at –5° and resuspended in acetone at –5°. This was repeated twice, and the powder (20–30 mg./g. of liver) dried at 0.1 mm. Hg (approx.) over P₂O₅, NaOH and paraffin wax, and stored in a desiccator below 0°. Clear extracts containing 'acetate-activating enzyme' were obtained by treating the powder for 20 min. at 0° with 5 mM-tris chloride buffer, pH 7.5 (1 ml./3–20 mg. of powder), and centrifuging at 12 000 g for 15 min.

Preparation media

The various media used in the preparation of cell fractions were as follows.

Salt medium. 0.10M-KCl; 0.05M-tris chloride buffer, pH 7.5; mM-EDTA; mM-ATP; 5 mM-MgSO₄.

Table 1. *Fractionation of chick-embryo homogenate*

Supernatants from the first and second centrifugings are designated *S_N* and *S_M* respectively. For description of media see text.

Salt medium		Sucrose media		Fraction	Symbol
Force (g)	Time (min.)	Force (g)	Time (min.)		
600	5	600	15	Nuclei + unbroken cells	<i>N</i>
8 500	10	12 000	10	Mitochondria	<i>M</i>
105 000	60	105 000	60	Microsomes	<i>P</i>
—	—	—	—	Final supernatant	<i>S_P</i>

Sucrose I. 0.20M-sucrose; 0.05M-tris chloride buffer, pH 7.5; mM-EDTA; mM-ATP; 5 mM-MgSO₄.

Sucrose II. 0.29M-sucrose; mM-EDTA; mM-ATP; 5 mM-MgSO₄.

Sucrose III. 0.31M-sucrose; mM-EDTA.

Sucrose IV. 0.31M-sucrose.

The salt medium was that found by Chappell & Perry (1954) to be most suitable for the preparation of pigeon-breast-muscle mitochondria, and might be expected to be applicable to the avian tissue used in the present work. Addition of EDTA to sucrose media used for preparation of muscle mitochondria was introduced by Slater & Cleland (1952). We find that fractionation of chick-embryo homogenates is much easier in salt solution and sucrose media I and II than in 0.31M-sucrose, since the particles do not adhere so tenaciously to glass and plastic surfaces as they do in the last-named medium and so are resuspended more easily.

Analytical methods and enzyme assays

Total nitrogen. This was determined by a micro-Kjeldahl method with incineration for 12 hr. Mitochondrial N was obtained by subtracting from the N content of the mitochondrial suspension that of the medium in which the mitochondria were suspended. A similar procedure was used for the other fractions.

Nucleic acids. These were determined by the Schmidt-Thannhauser procedure (Volkin & Cohn, 1954), except that their hydrolysis products were separated by paper chromatography. The following procedure was recommended by Dr Roy Markham, F.R.S., for whose advice and guidance we are very grateful. After removal of the acid-soluble compounds and lipids from the sample, the residue was treated with N-KOH to hydrolyse the RNA. The solution was then placed in portions on to Whatman no. 3MM paper so as to form a spot; after each addition an excess of 10N acetic acid was put round the periphery of the spot and the damp area dried with a jet of air; and after all additions had been made the spot was dried until no smell of acetic acid remained. The chromatogram was run with aq. 70% (v/v) propan-2-ol in an atmosphere of NH₃. Mononucleotides from the RNA were located by photography with transmitted u.v. light. The two spots so detected, which between them contain all four monoribonucleotides, were cut out and the material from each was eluted separately with 5 ml. of 0.1N-HCl. The extinctions of the two solutions were measured at 260 mμ in a Beckman model DU spectrophotometer against blanks from the paper, and their sum was used as a measure of the RNA. The DNA, which remained at the starting line of the chromatogram, was hydrolysed by treatment with conc. HCl, which was allowed to dry at room temperature. A chromatogram was then run with butanol (saturated with water)-formic acid (9:1, v/v). The adenine liberated from the DNA by the treatment with HCl was located as above and eluted with 5 ml. of 0.1N-HCl, and the extinction at 260 mμ was used as a measure of the DNA.

Succinate oxidase. This was assayed manometrically at 37° by the procedure of Schneider & Potter (1943), activity in the absence of added succinate being subtracted.

Adenosine triphosphatase. This was determined by measuring the inorganic phosphate released in 10 min. at 23° by 0.1 ml. of a mitochondrial suspension in a medium (total vol., 1 ml.) containing 0.05M-tris chloride buffer, pH 7.5, and 2 mM-ATP, and brought to 0.31 osmolar, with

sucrose when the mitochondria were prepared in sucrose-containing media, or with KCl when they were prepared in salt medium. The reaction mixture was deproteinized by addition of 1.8 ml. of 4.5% (w/v) trichloroacetic acid and filtration through Whatman no. 42 paper. Phosphate was determined in a 2 ml. portion by the method of Fiske & Subbarow (1925) with a Beckman model DU spectrophotometer at 650 mμ.

RESULTS

Characterization of cell fractions from 5-day embryos

Microscopic and staining studies. Under the phase-contrast microscope fraction *N* was seen to consist of debris and unbroken cells, small particles showing Brownian movement, and somewhat larger bodies which predominated and were identified as nuclei, since they could be stained by the Feulgen procedure. Small lighter areas within the stained nuclei presumably represented nucleoli. When prepared in 0.31M-sucrose the nuclei were 10–15 μ in diameter, but with ATP and Mg²⁺ ion in the medium only 4–5 μ. With lower concentrations of sucrose they swelled and frequently burst.

Fraction *M* under the phase-contrast microscope ($\times 1425$) appeared to consist almost entirely of refractile particles which were just visible and exhibited Brownian movement. They were stained blue by Janus green B. If an oxidizable substrate (succinate) were added and oxygen excluded, the stained particles became purple in 6 hr. and red in 24 hr. Such behaviour, however, does not identify them for certain as mitochondria, since separated cell particles other than mitochondria may take up and reduce this dye (Lazarow & Cooperstein, 1953).

Fraction *P* was difficult to resuspend, but the agglutinated masses seen under the microscope in no way resembled agglutinated mitochondria. The centrifuged *P* pellet was bluish and hyaline, whereas the *M* pellet was mushroom-coloured and opaque. Paigen (1956) has observed that mouse-liver microsomes adsorb haemoglobin. When haemoglobin was added to chick-embryo homogenate, the *P* pellet when isolated was bright red whereas no change was seen in the *M* pellet.

S_P appeared completely clear under the phase-contrast microscope.

To provide further evidence for the identification of the various cell fractions, the distributions between them of total nitrogen, RNA, DNA and succinate oxidase were determined for comparison with the findings with adult-rat liver.

Distribution of total nitrogen, ribonucleic acid and deoxyribonucleic acid. (Fig. 1). The distribution in cell fractions prepared both in salt medium and in sucrose medium I are seen to be similar to those found with adult-rat liver fractionated in sucrose solution (Schneider, 1948), except that (i) fraction *N* contained a higher proportion of the total

nitrogen in the embryo preparations than it did in those from adult-rat liver, and (ii) up to 4 % of the DNA was found in fraction *M* of embryo preparations. The DNA in *M* could be reduced to nothing if fraction *N* were spun down harder, but as the main object was to prepare mitochondria in good yield as rapidly as possible, the slight nuclear contamination was accepted.

Brachet & Jeener (1943-45) showed that, whereas in adult tissues most of the RNA was to be found in particles sedimenting at accelerations up

to 75 000 *g*, with frog oocytes a large proportion remained in the supernatant. The latter is not true of chick-embryo homogenates, in which most of the RNA is particulate.

Proportion of the succinate oxidase activity in the mitochondrial fraction. (Table 2). Fractions *N* and *S_M* were combined before the activity was measured, preliminary work having established that the activity in the combined fractions was equal to the sum of their separate activities. About 70 % of the activity of the homogenate was found in *M*, and about 25 % in the remaining fractions. Activity/mg. of nitrogen was many times greater in *M* than in the homogenate. Activity in fraction *N* was presumably due to unbroken tissue and mitochondria; on washing *N*, activity passed from this fraction into the wash fluid.

The above distribution and staining studies indicate that cell fractions similar to those obtained from adult tissues can be prepared from 5-day chick embryos.

Adenosine triphosphatase activity of mitochondria from 5-day embryos

It is well known (e.g. Kielley & Kielley, 1951; Lardy & Wellman, 1953) that rat-liver mitochondria, when freshly isolated by suitable techniques, have a low adenosine triphosphatase activity, which is greatly stimulated by DNP. If the particles are aged or subjected to treatment considered to be damaging, the adenosine triphosphatase activity in the absence of DNP increases, whereas that in the presence of DNP is not so great as it is with fresh, untreated mitochondria. The activities in the presence and absence of DNP may thus give some indication whether mitochondria have undergone serious deterioration as a result of an unsuitable preparation technique. We have applied these tests in an attempt to assess the condition of our mitochondria prepared in the various media (Table 3). It is seen that preparation in different media led to different enzymic activities, whereas the percentage increases in activity in the presence of DNP were all of the same order.

Fig. 1. Distribution of RNA, DNA and total nitrogen in cell fractions of chick embryo and rat liver. The RNA in each fraction is expressed as a percentage of the RNA in the homogenate, and 'recovery' is the sum of the percentage values of all the fractions. DNA and total nitrogen are treated similarly.

Table 2. *Proportion of the succinate oxidase activity in the mitochondrial fraction*

Chick embryo was fractionated in sucrose medium I. Values for rat liver are derived from the data of Hogeboom, Schneider & Pallade (1948).

Fraction	Chick embryo			Adult-rat liver		
	Nitrogen (%)	Succinate oxidase		Nitrogen (%)	Succinate oxidase	
		(%)	(μ l. of O_2 /mg. of N/hr.)		(%)	(μ l. of O_2 /mg. of N/hr.)
Homogenate	[100]	[100]	188	[100]	[100]	770
<i>M</i>	10.2	70.2	1273	26.0	71.8	2120
<i>N</i> + <i>S_M</i>	84.4	25.4	57	72.9	20.4	214
Sum of <i>M</i> and (<i>N</i> + <i>S_M</i>)	94.6	95.6	—	98.9	92.2	—

Table 3. *Adenosine triphosphatase activity of mitochondria from 5-day chick embryos in the presence and absence of 2:4-dinitrophenol*

For description of technique and media see Experimental section. Activities are given as μ moles of phosphate released/mg. of N/hr., means and standard errors being shown. In the columns headed % the activity in the presence of DNP is given as a percentage of that in its absence.

Preparation medium	No. of preparations	DNP absent (activity)	0.01 mM-DNP		0.1 mM-DNP	
			Activity	%	Activity	%
Salt	4	16.4 $\pm$ 2.4	26.0 $\pm$ 3.3	159	51.7 $\pm$ 5.5	315
Sucrose I	2	18.8 $\pm$ 0.15	26.7 $\pm$ 3.0	142	42.3 $\pm$ 3.7	225
Sucrose II	5	6.5 $\pm$ 0.4	10.5 $\pm$ 0.6	162	16.8 $\pm$ 1.7	258
Sucrose IV	2	3.4 $\pm$ 0.3	6.1 $\pm$ 0.6	179	11.8 $\pm$ 2.6	347

It is clear that activity in the absence of DNP was much higher with mitochondria prepared in salt medium and sucrose medium I, both of which contained tris, than in sucrose medium II, which did not. Again, it was found that mitochondria prepared in salt medium showed a higher rate of oxygen uptake/mg. of mitochondrial nitrogen with various substrates than those prepared in sucrose medium II (Table 1 of the next paper, Carey & Greville, 1959). The possibility had to be considered that the presence of tris in the medium was causing an error in the determination of mitochondrial nitrogen, giving a low value with consequent falsely high specific activity. It might then be expected that the yield of mitochondria, measured as nitrogen, would be lower in media containing tris than in those not. However, it was found that the average yield of mitochondria, in mg. of nitrogen/embryo, was 0.065 with media containing tris (25 preparations) and 0.064 with media free from tris (20 preparations). The possibility of error due to tris was tested more directly as follows: various volumes of a suspension of embryo mitochondria in 0.31 M-sucrose were diluted to 0.5 ml. with 0.31 M-sucrose, and 0.5 ml. of a solution containing 0.14 M-sucrose and 0.1 M-tris, pH 7.5, was added. The concentration of tris in these diluted suspensions was then the same as in salt medium and sucrose medium I. The nitrogen contents of the various suspensions, and of the tris solution diluted with an equal volume of 0.31 M-sucrose, were then determined. The mitochondrial-nitrogen content of each diluted suspension was calculated (a) from the difference between the nitrogen contents of diluted suspension and diluted tris solution, and (b) from the nitrogen content of the undiluted suspension. (a) was never less than (b), so it was concluded that the presence of tris did not lead to low values for mitochondrial nitrogen.

Cytochromes in mitochondria from 5-day embryos

It has been reported (Potter & DuBois, 1942; Yaoi, 1928-29) that cytochrome *c* cannot be detected in chick embryos at the stage used in the present study. The observation in the next paper

Table 4. *Cytochromes in mitochondria from 5-day chick embryos*

A suspension of mitochondria from 50 embryos was stored frozen. After thawing it was centrifuged (12 000 g, 15 min.) and $\text{Na}_2\text{S}_2\text{O}_4$ was added to the pellet. The spectrum was observed at room temperature with a Zeiss micro-spectroscope ocular (column i). The pellet was then suspended in sucrose medium II (1 ml.) and, after addition of glycerol (1 ml.) and a trace of $\text{Na}_2\text{S}_2\text{O}_4$, cooled in liquid air. The temperature was allowed to rise until devitrification occurred (Keilin & Hartree, 1949) and the spectrum was again observed (column ii). Values in parentheses are those given for liver mitochondria by Chance & Williams (1956).

Component	α -Band (m μ)	
	(i)	(ii)
<i>a</i>	605 (604-605)	603 (601)
<i>b</i>	565 (562-564)	563 (560)
<i>c</i> ₁	552 (553-554)	554 (554)
<i>c</i>	— (550)	549 (549)

(Carey & Greville, 1959) that the respiration of mitochondria from 5-day chick embryos is inhibited by cyanide suggests, on the other hand, that the hydrogen transport is mediated by the cytochrome chain as in other tissues. It was therefore worthwhile to examine our mitochondria spectroscopically for the presence of cytochromes. We thank Dr R. Hill, F.R.S., for permission to use his apparatus for this purpose, and Dr D. S. Bendall for guidance. Table 4 shows that the mitochondria contain cytochromes similar to those in liver mitochondria. Of the bands observed, the cytochrome *b* bands of the embryo were least like those of liver. With the embryonic material at room temperature, in addition to the band at 605 m μ bands were spread over the region 550-568, with peaks at 565 and 552 m μ . The latter peak probably corresponds to cytochrome *c*₁, the cytochrome *c* band being too faint to observe clearly, although at liquid-air temperatures it was clearly distinguishable, albeit relatively weak. Possibly some of the cytochrome *c* was lost from the particles when they were thawed after storage, and was discarded with the supernatant when they were centrifuged for the

observations. However, despite the uncertainty about the amount of cytochrome *c* relative to that of the other cytochromes, there seems no reason to doubt that the hydrogen transport in chick-embryo mitochondria proceeds by the usual cytochrome route.

'Acetate-activating enzyme' in liver from chick embryos and recently hatched chicks

In connexion with the study of fatty acid oxidation by mitochondria from embryonic tissue described in the next paper (Carey & Greville, 1959) it became of interest to examine these particles for the presence of the 'acetate-activating enzyme'. This enzyme, also called 'acetyl-coenzyme A synthetase' (Dixon & Webb, 1958) and 'acetic thiokinase' [see *Biochem. J.* (1956), **64**, 782], catalyses the formation of acetyl-CoA from acetate and CoA in the presence of ATP. The enzyme from yeast has been studied by Jones, Black, Flynn & Lipmann (1953) and by Lynen (1953), and that from heart muscle by Beinert, Green, Hele, Hift, Korff & Ramakrishnan (1953), Korff (1953) and Hele (1954). For purposes of enzyme assay, the reaction has usually been followed by measurement of acetohydroxamic acid formation in the presence of hydroxylamine, of citrate formation in the presence of oxaloacetate and condensing enzyme, or by reduction of coenzyme I in the presence of malate, malate dehydrogenase and condensing enzyme. Beinert *et al.* (1953), however, describe briefly an assay of the enzyme in which the formation of acetyl-CoA is followed by the increase in extinction at 232 m μ (Stadtman, 1953). It seemed that such a method would be very suitable for the study of the enzyme in embryonic tissue. A procedure was tried out with extracts of acetone-dried mitochondria from livers of 12- to 14-day chicks as source of enzyme (see Experimental section), the system shown in Table 5 being chosen after consideration of the findings of the various workers cited above. The rate of increase of extinction at 232 m μ ($\Delta E/\text{min.}$) was measured at room temperature in a Beckman model DU spectrophotometer. As ATP absorbs strongly at this wavelength, it was used in low concentration, and the reference cell contained all components of the reaction mixture except acetate. The reaction was started by addition of acetate solution to the experimental cell, an equal volume of water being added to the reference cell. Table 5 shows that CoA and ATP, as well as acetate, were essential for the activity, and that potassium chloride, magnesium sulphate and glutathione stimulated, in agreement with the various authors mentioned above. Sodium ions were inhibitory (cf. Korff, 1953): in a similar experiment to that in Table 5 the activity with the complete system was 7.01 units, and fell to 3.96

when the potassium acetate was replaced by an equivalent amount of the sodium salt. Fig. 2B shows that the system approached maximal velocity at a CoA concentration of 40 μM . With 22.5 μM , as used in the experiments so far described,

Table 5. Cofactor requirements of 'acetate-activating enzyme' in an extract of liver mitochondria from 12- to 14-day chicks

Complete system: 0.02M-tris chloride buffer, pH 8.5; 0.05M-KCl; 66 μM -ATP; 0.33 mM-MgSO₄; 0.5 mM-glutathione; 3.3 mM-potassium acetate; 22.5 μM -coenzyme A; extract of mitochondria (34 $\mu\text{g.}$ of N). Total vol., 3 ml. For other details see text.

System	Initial specific activity ($\Delta E_{232}/\text{mg. of N/hr.}$)
Complete	4.06
Without KCl	1.88
Without ATP	0
Without MgSO ₄	1.25
Without coenzyme A	0
Without glutathione	1.95
Without extract	0

Fig. 2. 'Acetate-activating enzyme' in extract of mitochondria of 12-day chick liver. (A) Relationship between initial velocity and enzyme concentration. The abscissa represents the amount of extract (as $\mu\text{g.}$ of nitrogen) present in the 3 ml. of reaction mixture; CoA, 45 μM ; other constituents were as given in Table 5. (B) Relationship between initial velocity and CoA concentration. The system contained, in 3 ml., extract from 1.2 mg. of acetone-dried powder; other constituents were as given in Table 5. (C) Progress curve. The system included, in 3 ml., extract containing 26 $\mu\text{g.}$ of nitrogen; CoA, 45 μM ; other constituents were as given in Table 5.

progress curves were not linear; but when the CoA concentration was raised to $45\text{ }\mu\text{M}$ linear progress curves resulted (Fig. 2C) and activity was proportional to enzyme concentration (Fig. 2A). With this CoA concentration an assay method was thus achieved. It is not claimed, however, that the concentrations of the other components of the system are necessarily at their optimum values.

The relationship between activity and pH was ascertained with the tris chloride system as buffer from pH 6.9 to 9.2. Although frozen extracts appeared to be stable, some inactivation occurred during storage at 0° . The activity at one pH value (8.4) was therefore determined at intervals during the experiment and the activity at other pH values multiplied by appropriate factors to relate them to the activity of the freshly prepared extract. The total decrease in activity at pH 8.4 was not greater than 20%. The corrected curve (Fig. 3) shows two maxima, approximately at pH 7.8 and 8.4. Both were also obvious in the curve showing activity uncorrected for inactivation. Jones *et al.* (1953), using phosphate buffer and the hydroxamic acid method of assay, found a single optimum between pH 7.2 and 7.8.

Fig. 3 also shows an activity-pH curve obtained similarly to the other with an extract of mitochondria from livers of 16-day chick embryos. The same two optimum pH values are seen. The specific activity ($\Delta E/\text{mg. of nitrogen/hr.}$) of the preparation from the embryos was considerably higher than that of the preparation from the hatched chicks (20.0 at pH 8.5 compared with 6.5). With another preparation from 16-day embryos the corresponding value was 20.8.

Fig. 3. 'Acetate-activating enzyme' in extracts from livers of 16-day chick embryos (○) and 12-14 day chicks (●); velocity-pH curves. The reaction mixture (3 ml.) contained embryo extract (9.6 $\mu\text{g.}$ of nitrogen) from 0.16 mg. of powder, or chick extract (26 $\mu\text{g.}$ of nitrogen) from 0.40 mg. of powder. CoA, $45\text{ }\mu\text{M}$; other constituents were as given in Table 5. For other details see text. The activity at all pH values was constant for the first 15-20 min.

DISCUSSION

Great care has been taken throughout this work to avoid damaging the mitochondria. That their adenosine triphosphatase activity was stimulated by DNP may be taken as an indication that, at any rate, gross maltreatment has not been inflicted. The degree of such stimulation varies from one tissue to another; for example, Holton, Hülsmann, Myers & Slater (1957) found that, with addition of magnesium chloride, the ratio of the adenosine triphosphatase activities in the presence and the absence of 0.1 mM-DNP was sixteen for liver mitochondria and two for heart sarcosomes. With embryo mitochondria we found ratios of 2.2-3.5 (Table 3). It should be remarked that in experiments with mitochondria prepared in sucrose medium IV the incubation mixture contained no added magnesium chloride, whereas with the mitochondria isolated in the other three media it contained 0.5 mM-magnesium chloride, contributed by the mitochondrial suspension. Some isolation media, particularly those containing tris, yielded mitochondria with a higher adenosine triphosphatase activity in the absence of DNP than did others. The reason for this is not known. In contrast, Myers & Slater (1957) found that the presence of tris in the isolation medium decreased the adenosine triphosphatase activity of liver mitochondria. In our experiments, the high activity shown by particles isolated in some media would not seem to indicate that those media were particularly injurious, since the stimulation of the activity by DNP was of the same order in all cases.

Embryo mitochondria isolated in salt medium had an adenosine triphosphatase activity in the presence of 0.1 mM-DNP of about 50 μmoles of phosphate/mg. of nitrogen/hr. This may be compared with activities of 70-150 found by Lardy & Wellman (1953) for liver mitochondria maximally stimulated by DNP. Heart sarcosomes are twice as active as liver mitochondria after such stimulation (Holton *et al.* 1957).

Our observations with embryo mitochondria might be criticized on the ground that the particles were isolated from a mixture of tissues. This possible criticism is met to some extent by experiments with subdivided embryos reported in the next paper (Carey & Greville, 1959).

SUMMARY

1. Differential centrifuging of homogenates of whole 5-day chick embryos has been shown by distribution and other studies to give rise to cell fractions similar to those obtained with adult tissues, namely nuclei and unbroken tissue, mitochondria, microsomes and a soluble fraction.

2. The adenosine triphosphatase activity of the mitochondria, isolated in several different media, was stimulated by 2:4-dinitrophenol; this suggests that the mitochondria were not seriously damaged.

3. The absorption bands of the cytochrome components in the embryo mitochondria corresponded quite closely to those observed in liver mitochondria.

4. 'Acetate-activating enzyme' activity has been studied in extracts of the mitochondrial fraction from the livers of young chicks and of 16-day chick embryos.

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Mitochondria from Embryonic Tissues of the Chick

2. METABOLIC ACTIVITIES

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Needham (1931) produced evidence that during development the chick embryo uses its energy stores in a sequence: utilization of carbohydrate precedes that of protein, which in turn precedes that of fat. Results consistent with the view that

carbohydrate is utilized in the early stages have been obtained in studies with chick embryos *in vitro*. Thus in the presence of glucose, embryos of 3-5 days' incubation show a high anaerobic glycolysis (Warburg, Posener & Negelein, 1924) and a respiratory quotient of unity (Dickens & Šimer, 1930, 1931; Needham, 1932*a*). Needham (1932*b*) concluded from measurements of am-

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