Direct Spectrophotometric Studies of the Kinetics of Oxidation and Reduction of Ubiquinone of Heart-Muscle Particles

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Spectrophotometric measurement of extinction changes associated with oxidation and reduction of the respiratory components of Keilin & Hartree heart-muscle particles has led to a clearer understanding of the reaction sequence and mechanism of action of these components (Chance, 1952*a*). In some cases it has been possible to distinguish between components whose reaction kinetics are either consistent or inconsistent with the rate of electron transfer through the system (Chance, 1952*a*, 1958). Although these methods have been applied to the ultraviolet region of the spectrum, for example to soluble enzymes (Chance & Harting, 1953; Harting & Chance, 1953), and to measurements of the 340 m μ band of reduced pyridine nucleotide in mitochondrial suspensions (Chance, 1954*a, b*; Chance & Williams, 1955*b*), their application to turbid heart-muscle preparations involves formidable technical difficulties. The arrangement of an 'end-on' photomultiplier placed very close to a cuvette containing dilute suspensions and the use of a high sensitivity to extinction changes (Chance, 1952*a*, 1954*a*, 1957*a*) have largely solved the problems discussed by Keilin & Hartree (1958), who encountered difficulties in the measurement of extinction changes of heart-muscle particles in the visible region of the spectrum with commercially available spectrophotometers.

In view of the intense interest in the possible role of ubiquinone (coenzyme Q) in electron transfer in various tissues and tissue fractions, more accurate and detailed information on the kinetics of its reduction and oxidation is needed. Up to this time, determinations of the ratio of oxidized to reduced forms of ubiquinone have been made by rapid stopping of the reaction either with cold methanol followed by a light-petroleum extraction (Pumphrey & Redfearn, 1960; Redfearn & Pumphrey, 1960) or by heat denaturation, followed by cyclohexane extraction (Crane, Lester, Widmer & Hatefi, 1959; Hatefi, Lester, Crane & Widmer, 1959).

Direct spectrophotometric measurement of reactions of ubiquinone at 275 m μ requires light-

sources of high intensity and stability. Further difficulties arise in providing optics of appropriate aperture and photosurfaces of adequate sensitivity, and in minimizing stray and scattered light. Also, extinction changes at 275 m μ occur rapidly upon addition of succinate to cyanide-treated heart-muscle particles. In this paper technical considerations involved in such measurements are described and ultraviolet-extinction changes are compared in both rate and extent with those computed from measurements of light-petroleum extracts of heart-muscle particles (Pumphrey & Redfearn, 1960).

Ubiquinone is the first component of heart-muscle particles to be studied simultaneously by direct spectrophotometry and by extraction from the particles. In heart-muscle and guinea-pig-kidney mitochondria assays of the changes of concentration of bound pyridine nucleotide (Chance & Williams, 1955*b*; Chance & Baltscheffsky, 1958*a*; Chance & Hollunger, 1960) have been correlated satisfactorily with chemical analyses (Klingenberg, Slenczka & Ritt, 1959).

We have shown that the ultraviolet-extinction changes measured in heart-muscle particles can be attributed to reduction of ubiquinone to the quinol. In terms of the kinetics and inhibitor-sensitivity of this reaction, our results confirm and expand those of Pumphrey & Redfearn (1959, 1960). A preliminary report of these studies is presented elsewhere (Chance & Redfearn, 1960).

EXPERIMENTAL

Materials

Horse heart is the most satisfactory material for chemical analysis because of its freedom from interfering pigments. Heart-muscle particles were obtained according to the general directions of Keilin & Hartree (1938, 1947) but with certain modifications, which are described in full in the appropriate references. The following terminology will be used: method M, particles prepared by grinding the mince in a mechanical mortar (Keilin & Hartree, 1938, 1947); method B, particles prepared by homogenizing the mince in a Waring Blendor (Chance, 1952*b*; elaborated by Pumphrey & Redfearn, 1960); method S, particles prepared from muscle mince washed with 0.9% NaCl soln. to remove cytochrome *c* (Tsou, 1952).

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Since the mechanism of action of ubiquinone does not appear to differ in the three preparations, a detailed description of their properties is not included.

Pure crystalline ubiquinone-50, m.p. 48.5°, was obtained from pig heart (Morton *et al.* 1958).

Phosphate buffers were prepared from KH_2PO_4 and Na_2HPO_4 .

Methods

Spectrophotometry. Application of a double-beam spectrophotometer (Chance, 1954*a*) to the measurement of small extinction changes of cytochromes in heart-muscle mitochondria has been described by Chance & Baltscheffsky (1958*b*). The electronic output of the instrument represents the difference between extinction changes of the particle suspension at two wavelengths. Light-beams of the required wavelengths, obtained from a pair of Bausch and Lomb 250 mm. focus grating monochromators, are flickered alternately through the sample, and changes at one (reference) wavelength compensate for non-specific changes at the other (measurement) wavelength. To study the kinetics of reduction of ubiquinone, the measurement wavelength was set at 275 μ and the reference wavelength at 300 μ (275–300 μ). To determine difference spectra, the reference wavelength was fixed at 300 μ and various wavelengths in the range 250–310 μ were used to measure the reduction of ubiquinone by addition of succinate to cyanide-treated heart-muscle particles. The spectrophotometer was illuminated with a 100 w Nestor arc lamp, the intensity of which was stabilized by a photoelectric control circuit in the same way as tungsten and mercury lamps (Chance, 1957*a*; Chance, Conrad & Legallais, 1958).

Accurate optical measurements at 275 μ are difficult because of the sharp increase of extinction and scattering observed with heart-muscle particles at wavelengths below 320 μ , and because extinction changes due to cytochromes in the Soret region may alter the amount of stray light falling upon the photocell.

The simplest test for the latter possibility is to use heart-muscle particles so highly concentrated that essentially no transmission occurs at 275 μ . It is then possible to observe spurious responses at this wavelength due to cytochrome changes in the Soret region. In experimental tests, the double-beam spectrophotometer gives decreasing responses with increasing concentrations of particles and no spurious responses were observed. Co-Ni filters, transmitting between 250 and 310 μ , were used to eliminate stray light, in determination of both extinction coefficients and difference spectra.

The extinction coefficient of ubiquinone is smaller without filters than with them, which is to be expected if some stray light passes through the monochromator. The significant fact is that the difference spectrum obtained without filters, and therefore in the presence of some stray light, agrees satisfactorily with that of ethanolic solutions of ubiquinone. This means that the absorption of stray light is not measurably changed by oxidation and reduction of cytochromes at other wavelengths, probably because the stray light has no sharp wavelength maxima. In addition, the largest changes due to cytochromes are in the region of the Soret band where both increases and decreases occur on reduction, and these changes apparently cancel out. This is important in determining difference spectra with the double-beam spectrophotometer and in

recording rapid kinetic changes with the regenerative flow apparatus because optimum signal-to-noise ratios can be obtained only without filters. The double-beam apparatus has the advantage that it accurately measures increments of specific extinction in spite of appreciable amounts of 'constant' stray light.

Respiratory activity. Respiratory activity was measured by a vibrating platinum microelectrode (Chance & Williams, 1955*a*) or, at temperatures below 20°, by a stationary electrode in a rotating cell, a method developed by G. R. Williams. The latter assembly combines a calomel or Ag-AgCl reference electrode and a platinum microelectrode. These dip into a cell rotating at 120 rev./min. At the specified temperatures, the concentration of heart-muscle particles was high enough to avoid diffusion of O_2 into the cuvette (Chance & Williams, 1955*a*).

Kinetic analysis. The reduction of ubiquinone was initiated by stirring succinate rapidly into an open cuvette of 1 cm. path, containing 1–2.5 ml. of heart-muscle particles. The mixing time was < 1 sec. (Chance, 1950). Experiments were conducted at 25° or at 10° (Table 3). Assays at the lower temperature are believed to be more reliable since the reduction of ubiquinone may be so rapid in active preparations at 25° that it is incorrectly recorded.

Methods of determining (i) the rates of oxidation and reduction of ubiquinone and (ii) the steady-state oxidation–reduction levels of endogenous ubiquinone are described elsewhere (Pumphrey & Redfearn, 1960; Redfearn & Pumphrey, 1960).

Determination of exogenous ubiquinone. A mixture of 0.2 ml. of heart-muscle particles (method B, 34.7 mg. of protein/ml.), 0.8 ml. of phosphate buffer (0.1 M, pH 7.4), 0.1 ml. of KCN (0.06 M) and 0.05 ml. of ubiquinone in ethanol was incubated at 26° with 0.2 ml. of 0.3 M-sodium succinate. The reaction was stopped and ubiquinone was extracted and assayed as described by Pumphrey & Redfearn (1960).

Kinetics of reduction of exogenous ubiquinone. The reaction mixture consisted of heart-muscle particles, buffer and KCN as above, together with 0.05 ml. of ethanolic ubiquinone (3.5 mM). The reaction was started by the addition of 0.2 ml. of 0.3 M-succinate. The reaction was stopped and the residual ubiquinone assayed as above.

Estimation of protein. The heart-muscle particles were diluted to between 1 and 10 mg. of protein/ml. and 1 ml. of the diluted particles was mixed with 0.5 ml. of water, 1.5 ml. of biuret reagent (Gornall, Bardawill & David, 1949) and 2 drops of 40% (w/v) sodium cholate. The mixture was incubated for 30 min. at room temperature, and the extinction at 540 μ then read in a spectrophotometer. The protein concentration in the diluted particles is given by $E_{540 \mu}/0.096$ mg. of protein/ml.

In the experiments of Figs. 1, 2*a* and 3, the simple relationship, fat-free dry weight = $1.3 \times E_{500 \mu}$ (Chance, 1952*b*), was used.

RESULTS

Optical data

A criterion for the measurement of a valid optical response. Addition of succinate to heart-muscle particles gives a readily measurable signal due to fumarate production (Chance, 1952*a, b*). A typical example of such an experiment is shown in Fig. 1.

Fig. 1. Kinetics of u.v.-extinction changes in heart-muscle particles. *Left.* Treated with succinate and then with cyanide. M-Succinate was added to a final concentration of 8 mM. The upward slope of the trace indicates increasing extinction, measured at $272-295\text{ m}\mu$, due to the formation of fumarate. M-Cyanide was added to a final concentration of 1.6 mM; after a small artifact caused by stirring, there was decreased extinction at $272\text{ m}\mu$ (downward slope of the trace). *Right.* Particles were pretreated with M-cyanide to a final concentration of 1.6 mM. After an artifact caused by stirring, no net change of extinction occurred. The addition of M-succinate to a final concentration of 1.0 mM was followed by a large decrease of extinction, measured at $272-295\text{ m}\mu$. Particles (method B, 0.5 mg. of protein/ml.) in 0.1 M-phosphate buffer, pH 7.2; temp. 26° ; Co-Ni filter. (Expt. 956b.)

Fig. 2a. Comparison of the magnitude of extinction changes caused by adding cyanide (O) to succinate-treated heart-muscle particles (0.42 mg. of protein/ml.) with the rate of extinction changes caused by adding succinate (●) and with the spectrum of pure fumarate (⊙). The left-hand scale of the ordinate corresponds to changes with cyanide for an optical path of 1 cm., the right-hand scale to the rate of change in extinction with succinate and to the extinction of pure fumarate. The peak observed in the presence of cyanide ($\sim 280\text{ m}\mu$) is close to the expected location for the extinction peak of ubiquinone. (Expt. 955.)

On the left the rise of the trace, corresponding to increasing extinction ($272-295\text{ m}\mu$), indicates production of fumarate. Addition of cyanide causes an abrupt downward deflexion, indicating a decrease of $E_{272\text{ m}\mu}$. On the right the preparation is pretreated with cyanide, which causes no extinction change other than that due to the stirring artifact (small dip and rise of the trace). On addition of succinate, there is an abrupt downward deflexion, which exceeds considerably that observed on the addition of cyanide (left). This suggests that the reaction is incomplete in the presence of succinate only. To determine the wavelength dependence of these effects the latter experiment was repeated at a succession of wavelengths (Fig. 2a, solid circles). Since concentrations of heart-muscle particles and of succinate were not varied in these experiments, the slope of the trace representing fumarate production should be proportional to the extinction coefficient of fumarate at the wavelengths used. To verify this, the rate of extinction change in the presence of succinate is plotted as a function of wavelength in the spectral region $250-300\text{ m}\mu$ (solid circles). The extinction rises at shorter wavelengths in a manner characteristic of the extinction spectrum of fumarate. A quantitative test of the shape of the fumarate spectrum is afforded by the circled points, which represent the spectrum of fumarate in solution (Racker, 1950). This result supports the conclusion that the shape of u.v. spectra can be measured without measurable effects from light-scattering and stray-light errors, and verifies that the oxidase activity of heart-muscle particles can be measured by the rate and extent of fumarate production (Chance, 1952b).

The difference spectrum of extinction changes that occur upon adding cyanide to succinate-treated heart-muscle particles is represented by the open circles of Fig. 2a. Values of the ordinate here correspond to the change of extinction for a 1 cm. optical path. The peak just below $280\text{ m}\mu$ is near the expected location for the peak of ubiquinone. Since this peak disappears upon reduction, the values of the ordinate are given a negative sign.

Activity criterion and effect of dilution of heart-muscle particles. It was desirable to have a standard value relating rate of fumarate production to concentration of material reducible by cyanide in succinate-treated heart-muscle particles. The left trace of Fig. 1 indicates the nature of this test, which consists merely of determining the quotient of the slope of the fumarate trace and the extinction change occurring on addition of cyanide to succinate-treated particles. For a given preparation this quotient should be constant, since it has the dimensions of enzymic activity (rate of produc-

tion of product divided by amount of enzyme, i.e. sec.^{-1}). Table 1 summarizes these values for a variety of dilutions of a heart-muscle suspension. Experimentally, the method is sufficiently sensitive for satisfactory measurements with heart-muscle particles diluted 167 times, at which a value of 210 is obtained. With dilutions of 130- and 86-fold slightly lower values are obtained, but, with a 60-fold dilution, a large discrepancy in the ratio is observed. However, dilutions between 167- and 86-fold (0.4–0.7 mg. of fat-free dry wt./ml.) are satisfactory for experimental studies at ~ 272 – $295 \text{ m}\mu$. The constancy of these values does not necessarily imply that extinction changes due to production of fumarate and reduction of ubiquinone are individually measured with accuracy, but rather that proportional errors may be made.

Comparison of difference spectra of ultraviolet-extinction changes with those of ubiquinone extracted with light petroleum. In Fig. 1 (left) extinction change observed on adding cyanide could conceivably have been due to a stray-light effect, light-leakage in the visible region being modified by reduction of cytochrome that occurs when cyanide is added to succinate-treated heart-muscle particles. However, since reduced forms of cytochromes absorb more light than oxidized forms, it is unlikely that reduction of cytochromes is responsible for the apparent decrease of extinction. Nevertheless it was considered important to make a detailed investigation of the nature of the u.v.-extinction band that disappears on reduction of the components in heart-muscle particles. For this reason we have repeated the experiment of Fig. 2a (solid trace) without Co-Ni filters (Fig. 2b) and have superimposed upon it the spectrum of an ethanolic solution of ubiquinone extracted from identical particles. These two spectra are normalized so that their maxima are superimposed. Although the shapes are similar, there is some displacement toward longer wavelengths of the difference spectrum for heart-muscle particles. There are three possible explanations for this. The first is that ethanol was the solvent for the difference spectrum of extracted ubiquinone, whereas the medium for endogenous ubiquinone might in effect be water or lipid. The second is that a certain amount of 'pulling' of the spectrum of endogenous ubiquinone towards longer wavelengths might be expected because (a) in this region extinction by protein components of heart-muscle particles increases rapidly with decreasing wavelengths and (b) there is increased light-scattering at shorter wavelengths. The third is that extinction changes due to haemoproteins might contribute on the long-wavelength side of the extinction band. Of these possibilities, the last is the most improbable: cytochrome c, for which quantitative data are

available (Margoliash & Frohwirt, 1959), shows the same changes of extinction upon oxidation and reduction at $275 \text{ m}\mu$ as at $300 \text{ m}\mu$. Between these two wavelengths, there would be a relative diminution of extinction in the transition from oxidized to reduced states, so that, at least for cytochrome c, any influence from the δ band is unlikely. We consider the first and second explanations for the shifted extinction peak more likely.

Table 1. *Effect of dilution of heart-muscle particles on extinction changes of ubiquinone (Expt. 955c)*

Heart-muscle particles (method B), 63 mg. of fat-free dry wt./ml., were used at the dilutions indicated; 0.1M-phosphate, pH 7.8. The reaction was initiated with M-succinate to a final concentration of 8 mM. After the rate of fumarate production had been measured, M-cyanide was added to a final concentration of 1.6 mM, causing the reduction of ubiquinone from the steady state to the fully reduced state. The ratio of the rate of fumarate production to the extinction change of ubiquinone in response to the addition of cyanide is termed the 'activity value'.

Dilution	167	130	86	60
Concn. (mg./ml.)	0.4	0.5	0.7	1.0
Activity value (sec.^{-1})	210	180	180	320

Fig. 2b. Superposition of extinction changes observed on addition of succinate to cyanide-treated heart-muscle particles (0.8 mg. of protein/ml.) (○) and those recorded for an ethanolic solution of ubiquinone extracted from identical particles (●). The latter spectrum was obtained by the indirect method described by Pumphrey & Redfearn (1960), the former by the direct method. Heart-muscle particles (method B) were diluted in 0.15M-phosphate buffer (pH 7.8) for 30 sec.; M-cyanide was added to a final concentration of 1.2 mM; M-succinate was added to a final concentration of 4 mM. Temp. 26° ; Co-Ni filters were not used. (Expt. 987a.)

The first is particularly applicable because ubiquinone absorbs at a longer wavelength in aqueous suspension than it does in ethanol (B. Chance & E. R. Redfearn, 1959, unpublished work). In fact, it is possible that in the heart-muscle particles ubiquinone absorbs at a wavelength differing from that of ubiquinone in any known solvent.

Extinction coefficient of ubiquinone. The extinction change observed on reducing ubiquinone with succinate in the presence of cyanide in heart-muscle particles is less than that computed from the chemically determined ubiquinone content of particles having the same protein concentration (Table 2). Further experiments were therefore made to determine the effective value of the extinction coefficient of a solution of ubiquinone under both sets of experimental conditions. According to Pumphrey & Redfearn (1960), the change of molar extinction coefficient corresponding to the reduction of ubiquinone dissolved in ethanol and measured at 275–300 $m\mu$ is $13 \text{ cm.}^{-1} \text{ mm}^{-1}$. This value was calculated from the absolute absorption spectra of ubiquinone and the quinol. A value of 13 is also obtained in the apparatus we use, but if ubiquinone is dispersed in water the extinction coefficient is less (Table 2). If an ethanolic solution of ubiquinone is added to heart-muscle particles and Co–Ni filters are used, the extinction coefficient is between 5 and $8 \text{ cm.}^{-1} \text{ mm}^{-1}$. Under these conditions, the extinction change due to reduction of endogenous ubiquinone divided by the concentration of ubiquinone obtained on analysis of an extract gives a coefficient of $\sim 6 \text{ cm.}^{-1} \text{ mm}^{-1}$ for a 1 cm. path cuvette and the particular optical geometry described under Methods. Since there are uncertainties in the completeness of the indirect extraction method and in optical calibration obtained by adding a suspension of ubiquinone to the heart-muscle particles, we have arbitrarily chosen a value of $10 \text{ cm.}^{-1} \text{ mm}^{-1}$ for rate calculations in this paper.

Extinction coefficient of fumarate. As a further check upon the accuracy of the spectrophotometric method, the extinction coefficient of fumarate was determined by quantitative reduction of the oxygen dissolved in the air-saturated buffer solution of heart-muscle particles with formation of a corresponding concentration of fumarate. The fumarase activity of these particles is negligible compared with the succinic-oxidase activity. For the reaction to be complete within a reasonable time, a relatively high concentration of particles is used (0.5 mg./ml.), and this requires an optical path of 2 mm. rather than 1 cm. for accurate measurement of the large extinction change. A typical record appears in Fig. 3, where the extinction change corresponding to the formation of fumarate is 0.0356. Taking the solubility of oxygen

in phosphate buffer to be $240 \mu\text{M}$ (Chance & Williams, 1955a), the extinction coefficient is calculated to be $0.37 \text{ cm.}^{-1} \text{ mm}^{-1}$.

Titrimetric and inhibition data

Effect of succinate concentration on the reduction of ubiquinone. In some heart-muscle-particle preparations, the rate of reduction of ubiquinone on adding succinate was sufficiently slow that it could be measured at 26° (cf. Figs. 1, 4 and 5). Fig. 4 illustrates the effect of two concentrations of succinate on the rate and extent of reduction of ubiquinone in cyanide-treated heart-muscle particles. A straight line is obtained when reciprocals of the rates are plotted against reciprocals of succinate concentration within the range 0.030–1.00 mm-succinate. The succinate affinity for ubiquinone reduction is 0.5 mm and that for half-maximal respiration, as

Table 2. Millimolar extinction coefficients
(Expt. 990)

The millimolar extinction coefficients of endogenous and added ubiquinone were determined under the following conditions: particles (method B), were diluted to 0.8 mg. of protein/ml. in 0.1M-phosphate, pH 7.2. They were treated at 26° with m-cyanide to a final concentration of 1.2 mm and then with m-succinate to a final concentration of 4 mm. The extinction change was measured at 275–300 $m\mu$, with Co–Ni filters. Ethanolic ubiquinone (0.5 mm) was added to a final concentration of $1.85 \mu\text{M}$ and the change measured again. Extinction coefficients were determined as described in the text.

Fig. 3. Determination of a molar extinction coefficient for fumarate in succinate-treated heart-muscle particles. The downward deflexion of the trace indicates fumarate production and an increase of extinction at 275–300 $m\mu$. Particles (method B, 0.5 mg. of protein/ml.) in 0.1M-phosphate buffer were equilibrated with air at 26° . (Expt. 96–95b.)

measured by the platinum microelectrode, is also 0.5 mM (Chance, 1952*b*).

Effect of antimycin A. Pumphrey & Redfearn (1960) found that both the rate and the extent of reduction of ubiquinone in heart-muscle particles were unaffected by antimycin A. Since this is a crucial observation in interpreting the locus of action of ubiquinone, we have repeated the experiment spectrophotometrically (Fig. 5). When

4 mM-succinate is added to cyanide-treated heart-muscle particles (left), the spectrophotometric trace shows an upward deflexion, corresponding to a decrease in extinction at 275–300 m μ . If, instead of cyanide, sufficient antimycin A is added to inhibit respiration completely (right), subsequent addition of succinate also causes an upward deflexion. In this case, the rate of change of extinction is only 4% less than that observed with

Fig. 4. Effect of succinate concentration on the rate of extinction change of heart-muscle particles, measured at 272–295 m μ . The particles were pretreated with m-cyanide to a final concentration of 1.6 mM. Succinate was added to a final concentration of 1.2 mM (left) and 0.12 mM (right). The experimental conditions were identical with those of Fig. 1. (Expt. 956.)

Fig. 5. Comparison of the kinetics and extent of reduction of ubiquinone in cyanide-inhibited and antimycin-inhibited heart-muscle particles. *Left.* Particles were pretreated for ~30 sec. with m-cyanide to a final concentration of 1.2 mM and then with m-succinate to a final concentration of 4 mM. The rise of the trace indicates decreased extinction at 275–300 m μ , and the reduction of ubiquinone. *Right.* Particles were pretreated for ~30 sec. with antimycin A (1 mg. dissolved in ethanol) to a final concentration of 2 μ M and then with m-succinate to a final concentration of 4 mM. The experimental conditions were as in Fig. 2*b*. (Expt. 990*b*-11, 12.)

cyanide-treated particles. The equality, within experimental error, of the changes of extinction in the two experiments is of particular interest. It appears that all the u.v.-absorbing material can be reduced as effectively in the presence of antimycin A as in the presence of cyanide. Chemical assays also indicate that all the petroleum-extractable ubiquinone is reduced under these conditions (Pumphrey & Redfearn, 1960). Thus it seems reasonable to conclude that the quinone acts on the substrate side of the antimycin-A-sensitive point and that no detectable amount operates between cytochrome c_1 and oxygen.

Kinetic data

Nature of the reaction kinetics. Examination of the reduction kinetics, as illustrated particularly in Figs. 4 and 5, shows that the reaction is zero order for the first two-thirds of its course. No measurable 'jump' in the reaction kinetics occurs on addition of succinate. For this reason, rates of reduction are computed on the basis of the slope of the linear portion of the traces. It is a matter of some concern that chemical extraction gives results which appear to fit first-order kinetics (Pumphrey & Redfearn, 1960; Chance, 1960).

Effect of adding ubiquinone to heart-muscle particles. In view of the considerable interest in reactivation of electron transfer in solvent-extracted heart-muscle particles (Green, 1959), we have determined the spectrophotometric response of particles to added solutions of ubiquinone. Fig. 6 illustrates the reduction of endogenous ubiquinone by addition of succinate to cyanide-treated particles. When reduction is practically complete, ethanolic ubiquinone is added to a final concentration of $1.85 \mu\text{M}$. The abrupt rise of the trace is due to extinction of the added oxidized ubiquinone, but the ensuing horizontal trace shows that this ubiquinone remains oxidized for at least 30 sec., whereas the endogenous ubiquinone was reduced in a few seconds.

This result was confirmed by chemical studies in which the time scale was extended beyond 30 sec. Only 25 % of the total ubiquinone was reduced in 1 min., and less than 50 % in 5 min. The concentration of the added ubiquinone ($134 \mu\text{M}$) was about six times that of the endogenous ubiquinone and therefore represented a more favourable condition for a rapid reduction reaction than in the spectrophotometric study of Fig. 6.

It is thus apparent that there is little relationship between the activities of the respiratory chain with respect to endogenous ubiquinone and to added ubiquinone.

Kinetics of reduction of endogenous ubiquinone: comparison of indirect chemical and direct spectro-

scopic methods. In several experiments spectroscopic and chemical methods were compared in the same preparations under nearly identical conditions. The ubiquinone-reductase activities are compared with the succinic-oxidase activities in Table 3. The values in the Table indicate, as was found previously (Pumphrey & Redfearn, 1959; Redfearn & Pumphrey, 1960), that the rate of reduction of ubiquinone by succinate is only a fraction of the succinic-oxidase rate. The validity of this conclusion is considered in more detail in the Discussion.

Comparison of rate of fumarate production with rate of ubiquinone reduction. Experiments such as those of Fig. 1 give rates of fumarate production and ubiquinone reduction under identical conditions. In Fig. 1 the ratio of the rate of extinction change of ubiquinone to that of fumarate is 7. An experiment in which an attempt was made to obtain a higher accuracy is illustrated by Fig. 7. Here the temperature was 8° and the optical path was 2 mm. Under these conditions, a higher concentration of heart-muscle particles was permissible and the rate

Fig. 6. Comparison of the rates of reduction of endogenous and exogenous ubiquinone in heart-muscle particles. The particles were pretreated with *m*-cyanide for 30 sec. to a final concentration of 1.2 mM and then with *m*-succinate to a final concentration of 4 mM . The reduction of ubiquinone is indicated by the downward deflexion of the spectrophotometric trace (decreased extinction at $275-300 \text{ m}\mu$). When the reaction was substantially complete, 0.5 ml. of ethanolic ubiquinone was added to a final concentration of $1.85 \mu\text{M}$. The upward deflexion of the trace corresponds to increased extinction of oxidized ubiquinone. The horizontal portion of the trace indicates that ubiquinone was not significantly reduced. The Co-Ni filter was used; conditions otherwise were as in Fig. 2b. (Expt. 990b.)

Table 3. Succinate-ubiquinone reductase rates in heart-muscle particles

Details of the spectrophotometric experiments are given in the Figures, legends and text. The experimental data have been multiplied by the dilution factor for the particular experiment so that they will be comparable with the results calculated from the extraction method. The values can be converted into $\mu\text{moles}/\text{mg.}/\text{hr.}$ by multiplying by 3.6 and dividing by the protein concentration. Particle suspensions were diluted 30-40-fold: activities are given for undiluted preparations. (Expts. 994, 996 and 998.)

Method	Concn. of protein (mg./ml.)	[Ubiquinone] ($\mu\text{moles/g.}$ of protein)	Age of prep. (days)	Rate of reduction by succinate			Rate of oxidation of succinate			Ubiquinone reductase Succinic oxidase
				By extraction method ($\mu\text{moles/l.}/\text{sec.}$)	Temp.	By spectrophotometric method ($\mu\text{moles/l.}/\text{sec.}$)	Temp.	O ₂ electrode method ($\mu\text{moles/l.}/\text{sec.}$)	Temp.	
M	34.5	3.5	1	—	—	—	—	364	25°	—
M	34.5	3.5	5	7.2	0°	9.1	—	31	7	0.29
M	34.5	3.5	7	8.1	7	10	7°	15.5	8.5	0.58
B	29.6	3.5	12	22	7	27	9.5	73	7	0.34
M	34.2	3.3	2	10.4	7	7.4	7	40	8.5	0.22

of production of fumarate was rapid even at the low temperature. The ratio of rates of extinction changes was now 7.5 or 9.0 depending upon the order of addition of succinate and cyanide.

Steady state. Fig. 1 clearly indicates that the extinction change obtained on adding succinate to cyanide-treated particles exceeds that obtained on adding cyanide to succinate-treated particles. If this is considered evidence of partial reduction of ubiquinone in the steady state of succinate utilization in heart-muscle particles, the percentage reduction of ubiquinone under these conditions was ~30% (Table 4). In general, the extent of reduction found chemically considerably exceeded that measured optically, at both room and low temperatures. The cause of this discrepancy is not known. However, in a cytochrome c-deficient preparation (method S), the values are in reasonable agreement.

DISCUSSION

The fundamental result of these experiments is that a decrease of u.v. extinction occurs upon addition of succinate to either cyanide-free or cyanide-inhibited heart-muscle particles but that the decrease is considerably greater in the presence of the inhibitor. The succinate concentration required for half-maximal change of extinction is roughly the same as that required for half-maximal respiratory activity. Upon addition of oxygen to anaerobic, succinate-reduced heart-muscle particles, u.v. extinction increases rapidly, later subsiding at a rate consistent with the rate of oxygen exhaustion by the system. Although these observations suggest that a component of the respiratory chain is being studied (Green, 1959), a more detailed investigation is required if this point is to be established.

A quantitative study of the kinetics of the change of u.v. extinction necessitates (i) identification of the substance involved, (ii) an estimate of its molar extinction coefficient, (iii) determination of its site of action in the respiratory chain, and (iv) comparison of the rates of its oxidation and reduction with the rate of electron transfer in the cytochrome chain.

(i) Interpretation of the u.v.-extinction change as a reduction of ubiquinone depends upon satisfactory agreement between the change caused by succinate addition to intact heart-muscle particles described in this paper and that caused by addition of sodium borohydride to a light-petroleum extract of ubiquinone from identical particles (Pumphrey & Redfearn, 1960).

The possibility might be considered that ubiquinone is reduced to a free radical or semiquinone (Michaelis & Schubert, 1938) which could mediate

Fig. 7. Comparison of the rates of change of extinction in heart-muscle particles caused by the production of fumarate (a) in the presence of succinate (upward slope of the trace) with the rate of reduction of ubiquinone after cyanide addition or (b) with the rate of reduction of ubiquinone upon addition of succinate to cyanide-treated particles. Particles (method B, 0.39 mg. of protein/ml.) in 0.1 M-phosphate buffer, pH 7.4; temp. 8°. (Expt. 95.)

Table 4. *Steady-state oxidation-reduction levels of endogenous ubiquinone*

Experiments were carried out as described in the text and Figure legends. For the spectrophotometric experiments, the percentage reduction was calculated from the ratio of the recorder deflexions obtained on adding succinate to cyanide-free heart-muscle particles to the sum of this deflexion and that obtained on adding succinate to cyanide-inhibited particles. For the extraction method, the values were obtained by adding first succinate and then cyanide to heart-muscle particles.

Particle preparation	Age of prep. (days)	Temp.	Steady-state reduced ubiquinone (%)	
			Extraction method	Spectrophotometric method
B	8	9.5°	35	13
B	2	26	50	—
M	2	7	47	29
M	3	7	55	25
M	3	9.5	—	35
S	5	26	73	88

between two-electron steps in flavins and one-electron steps in cytochromes (Chance & Williams, 1956). However, the close correspondence of the difference spectra of solutions of ubiquinone with those of heart-muscle particles provides no evidence for such an intermediate. These observations also make it unlikely that a spectroscopically distinct ubiquinone-phosphate compound is present at measurable concentrations in the non-phosphorylating particles.

(ii) Although a precise determination of the molar extinction coefficient of ubiquinone in the heart-muscle particles is not yet possible, an approximate value can be suggested. The extinction coefficients measured in ethanol or in water in the double-beam spectrophotometer agree with those measured in the Beckman DU spectrophotometer

in ethanol or in water. However, the extinction coefficient in water is much less than that in ethanol and agrees with the coefficient computed from addition of known concentrations of ubiquinone to a water suspension of heart-muscle particles. This value in turn agrees with the extinction coefficient of endogenous ubiquinone calculated from the quotient of the extinction change, measured by addition of succinate and cyanide to the particles, and the concentration obtained by analysis of an extract of the particles. Although the coefficient for endogenous ubiquinone may be as low as $\sim 6 \text{ cm}^{-1} \text{ mm}^{-1}$ we have concluded that a value of $10 \text{ cm}^{-1} \text{ mm}^{-1}$ represents as good a value as the data warrant at the present time. It is obvious that the rates of reduction of ubiquinone given here would be increased

proportionally if $6 \text{ cm.}^{-1} \text{ mm}^{-1}$ were subsequently found to be a more nearly correct value for the extinction coefficient.

(iii) Experiments with antimycin A indicate that ubiquinone is on the substrate side of the antimycin-sensitive point in the respiratory chain. The extinction change at $275 \text{ m}\mu$ that accompanies succinate reduction in the presence of cyanide is no greater than in the presence of antimycin A. Nor does addition of cyanide to a system blocked with antimycin A and reduced with succinate cause any further increase of extinction (B. Chance, 1959, unpublished work). These results suggest, contrary to the postulates of Martius (1955) on the general function of quinones and those of Green (1959) on the role of ubiquinone in particular, that no measurable amount of ubiquinone participates between the antimycin-sensitive point and oxygen. These results confirm previous titrations of the respiratory chain with reduced DPN (Chance, 1957b). Those titrations showed that ubiquinone does not act as an intercytochrome carrier but the possibility that ubiquinone participates near the substrate end of the chain (Slater, 1957) was not eliminated. The remarkable inactivity of heart-muscle particles in the reduction of added ubiquinone suggests that high reductase activities towards exogenous ubiquinone can be obtained only by structural alteration of the particles to enhance accessibility of the reductase (Bendall & de Duve, 1960).

(iv) Correlations of ubiquinone and oxygen reduction activities have been made on various preparations. Results observed with our spectrophotometric or 'direct' method can now be compared with those reported by Redfearn & Pumphrey (1960) for the chemical extraction or 'indirect' method. Since none of the assays given here was made in the presence of added cytochrome *c*, the comparison is limited to data in Table 3 of Redfearn & Pumphrey (1960). It is apparent that in the absence of added cytochrome *c*, the succinic-oxidase values in this paper, measured at 25° , are considerably greater than those measured at 21° by Redfearn & Pumphrey. For example, an experiment at room temperature (Table 3, first row), suitably corrected, gives $38 \mu\text{moles/mg. of protein/hr.}$, whereas Redfearn & Pumphrey report values between 3.5 and $6.9 \mu\text{moles/mg. of protein/hr.}$ Thus the values in this paper pertain to much more active preparations.

An activity of $38 \mu\text{moles/mg. of protein/hr.}$ corresponds to $11 \mu\text{moles/g./sec.}$ With a ubiquinone concentration of $4 \mu\text{moles/g.}$, this amount would be turned over in $4/11 = 0.36 \text{ sec.}$ at 25° , provided it were in the main pathway of electron transfer. This time is too short for manual mixing, i.e. indirect, methods at room temperature. It is important to

note that dilution of the heart-muscle particles will not increase the time available for the measurement of ubiquinone reduction because this is a bound enzyme and its turnover time is independent of dilution (Chance, 1952a). For these reasons Table 3 includes four measurements of ubiquinone-reductase activities at $7-9.5^\circ$ where the time, as calculated above, ranges from 8 to 1.4 sec. , the latter being just satisfactory.

The ubiquinone-reductase activities are computed from the initial slopes of the reaction kinetics as measured by the indirect chemical extraction method or by the direct spectroscopic method with a value of $\Delta\epsilon_{275-300 \text{ m}\mu}$ of $10 \text{ cm.}^{-1} \text{ mm}^{-1}$. The three values obtained in the temperature range $7-9.5^\circ$ agree to within 20% , which is considered highly satisfactory for present purposes.

The ratios of the ubiquinone-reductase activities (means of direct and indirect methods) to the succinic-oxidase activities measured polarographically have a mean value of 0.36 . Thus the ubiquinone-reductase activity would appear to be about one-third of the succinic-oxidase activity.

The agreement between determinations of the steady-state reduction of ubiquinone by indirect and direct measurements is not as close as desirable, the latter method giving smaller values. Direct measurement of steady-state reduction was made approximately 30 sec. after adding succinate to the heart-muscle particles, whereas with the indirect method 6 min. elapsed between the addition of succinate and the measurement. The former is probably more relevant to reductase activity and the latter to the steady-state oxidase activity. A method which would compare the two under the same conditions is highly desirable.

In the experiments of Figs. 1 and 7 we have compared the rate of reduction of ubiquinone with the rate of formation of fumarate under very similar conditions: the particle suspensions and temperatures are identical and the times are almost the same. If one assumes agreement between the two rates, the ratio of the rates of extinction change should be equal to the ratio of the millimolar extinction coefficients.

$$\text{If } -\frac{d[Q]}{dt} = \frac{d[\text{fum}]}{dt} \quad (1)$$

$$\text{then } \frac{d(\Delta E_Q/\Delta\epsilon_Q)}{dt} = \frac{d(\Delta E_{\text{fum}}/\Delta\epsilon_{\text{fum}})}{dt} \quad (2)$$

and, since values of $\Delta\epsilon$ are not functions of time,

$$\frac{\frac{d(\Delta E_Q)}{dt}}{\frac{d(\Delta E_{\text{fum}})}{dt}} = \frac{\Delta\epsilon_Q}{\Delta\epsilon_{\text{fum}}} \quad (3)$$

where *Q* represents ubiquinone, and *fum* represents fumarate.

Fig. 7 illustrates an experiment in which the ratio of the rates of change of extinction was 9 and the millimolar extinction coefficient of fumarate ($\Delta\epsilon_{\text{fum}}$) was found to be $0.37 \text{ cm}^{-1} \text{ mm}^{-1}$ by independent calibrations. From equation (3), $\Delta\epsilon_Q$ is $3.3 \text{ cm}^{-1} \text{ mm}^{-1}$. If the actual value of $\Delta\epsilon$ is $10 \text{ cm}^{-1} \text{ mm}^{-1}$ then the ratio of the rate of ubiquinone reduction to the rate of fumarate production is 0.33 as compared with the value 0.36 given above. Similarly, if $\Delta\epsilon$ is $6 \text{ cm}^{-1} \text{ mm}^{-1}$ the ratio is 0.66 and a value for $\Delta\epsilon_Q$ of 3.3 would be required to give equal rates of ubiquinone reduction and fumarate production.

If participation in electron transfer requires an initial rate of ubiquinone reduction at least as great as the steady-state rate of fumarate production, these results indicate that ubiquinone is not in the main electron-transport pathway.

So far we have tacitly assumed that, as a basis for comparison of the oxidase and reductase activities of ubiquinone with the steady-state rate of oxygen utilization, the initial rate of reduction of ubiquinone must be at least equal to the steady-state rate of reduction, i.e.

$$\frac{d[\text{QH}_2]}{dt} = -\frac{1}{2} \frac{d[\text{O}_2]}{dt} = \frac{d[\text{fum}]}{dt} \quad (4)$$

where QH_2 is reduced ubiquinone. This is appropriate only if the measured rates of change of ubiquinone are unaffected by 'back' reactions. For example, in the reduction of ubiquinone by flavoprotein,

$$\frac{d[\text{QH}_2]}{dt} = k_1[\text{Q}][\text{rfp}] \quad (5)$$

where rfp represents reduced flavoprotein, a 'back' reaction would be provided by the reoxidation of reduced ubiquinone by the adjacent oxidized component, especially since this component is fully oxidized at the time of addition of succinate:

$$\frac{d[\text{QH}_2]}{dt} = k_1[\text{Q}][\text{rfp}] - k_2[\text{QH}_2][\text{oxidant}]. \quad (6)$$

Thus the maximal rate of reduction of ubiquinone may not be observed under the experimental conditions, where it is difficult to measure the reaction before a small amount of reduced ubiquinone has been formed. In order to keep the amount of oxidant as low as possible, the kinetics of reduction have usually been measured in the presence of cyanide. However, adjacent members of the respiratory chain on the oxygen side of ubiquinone may act as oxidants. Their concentration is reduced by the use of antimycin A as an inhibitor, but no measurable change of rate is observed (Fig. 5). This result suggests either that (i) the 'back' reaction does not significantly affect the kinetics or (ii) antimycin treatment does not completely eliminate the store of oxidizing equivalents. The

fact that cytochrome *b* is so much more rapidly reduced in the antimycin-treated material (Chance, 1958) suggests that it can act as an oxidant of reduced ubiquinone under these conditions. Thus, as the reduction of ubiquinone is still opposed by a 'back' reaction in the presence of antimycin A, alternative (ii) appears to be the more acceptable. However, the fact that the stoichiometric ratio of ubiquinone to cytochrome *b* is < 4 weakens this argument: insufficient cytochrome *b* is present in the respiratory chain to oppose the reduction of ubiquinone during the entire course of the reaction. Thus the reduction kinetics in the presence of antimycin A would largely represent the unopposed reduction of ubiquinone and this favours the first alternative. This conclusion is supported by Fig. 5, which shows that the reduction kinetics of ubiquinone are zero order to the same extent in the presence of antimycin A as in the presence of cyanide.

Before the role of ubiquinone in electron transport can be elucidated, several questions raised by these kinetic studies must be answered. First, it will be essential to find an explanation of the difference between the maximum rate at which electron transport can occur in the steady state and the maximum rate at which electrons are transferred to ubiquinone. Secondly, the possibility that an immeasurably small amount of ubiquinone is reduced as rapidly as succinate is oxidized must be considered. Thirdly, it will be necessary to explain the zero-order kinetics of reduction of ubiquinone over such a large part of the reaction. Experiments bearing upon this problem have been published elsewhere (Chance, 1960). Finally, for a complete analysis, quantitative comparisons between ubiquinone and the other components of the respiratory chain, in terms of oxidation and reduction kinetics and of steady-state levels, must be obtained.

This paper, while extensive in its terms of reference, covers only a few of the many possible mechanisms of electron transfer and other mechanisms could make different demands upon the ubiquinone kinetics.

SUMMARY

1. An ultraviolet-extinction change is caused by addition of cyanide or antimycin A to a succinic-oxidase system. The magnitude of the change is proportional to the rate of production of fumarate.

2. The spectrum representing the extinction change caused by addition of cyanide to the succinic-oxidase system has a peak near $275 \text{ m}\mu$ and a shape resembling the difference spectrum of ethanolic solutions of reduced and oxidized ubiquinone derived from heart-muscle particles. The

former spectrum is, however, shifted to longer wavelengths.

3. The ultraviolet-extinction change is identified with the reduction of endogenous ubiquinone. No spectroscopic evidence for a semiquinone, free radical or otherwise altered reduction product has been obtained.

4. The extinction change is incomplete in the presence of succinate alone, but goes rapidly to completion in the presence of cyanide or antimycin A. About 0.5 mM-succinate gives half-maximal reduction of ubiquinone in the cyanide-inhibited system.

5. Shortly after the addition of succinate to the succinic-oxidase system, the reduction of ubiquinone is about 30% of the total reduction obtained in the presence of cyanide. This represents a steady-state reduction of ubiquinone that is less than that observed by the chemical extraction method and at longer times after adding succinate.

6. No spectroscopically detectable ubiquinone is found on the oxygen side of the antimycin-sensitive point, and insufficient ubiquinone is available to act as an 'intercytochrome carrier'.

7. Upon adding succinate to cyanide- or antimycin-treated particles, quinone is reduced in what appears to be a zero-order reaction for the first two-thirds of the reaction. The high activity of the heart-muscle preparations precluded accurate ubiquinone-reductase activity measurements by manual mixing at 26° and studies in the temperature range 7–9.5° are reported.

8. The kinetics of reduction of endogenous ubiquinone (derived from the direct spectrophotometric data) agree to within 20% with those obtained by chemical assay on light-petroleum extracts of ubiquinone. The direct data are computed on the basis of $\Delta\epsilon_{275-300\text{ m}\mu} = 10\text{ cm}^{-1}\text{ mM}^{-1}$.

9. The direct spectrophotometric and indirect chemical methods give ubiquinone-reductase rates at 7–9.5° that are about one-third of the succinic-oxidase rates.

10. A comparison of the reduction of ubiquinone and the production of fumarate made by the spectrophotometric method at 8° shows that the maximum rate of ubiquinone reduction is one-third that of the steady-state rate of fumarate production. It follows that only if the value of $\Delta\epsilon_{275-300\text{ m}\mu}$ for ubiquinone were $3.3\text{ cm}^{-1}\text{ mM}^{-1}$ would the rates of ubiquinone reduction and fumarate production be equal.

11. The effect of the 'back' oxidation reaction upon ubiquinone-reductase activity is shown to be insignificant.

12. On the basis of the chemical mechanism proposed here the kinetic discrepancies between ubiquinone-reductase activity and succinic-oxidase activity are irreconcilable, and so far no part of the

total ubiquinone has been found to be reduced at a rate equal to that at which succinate is oxidized in the steady state.

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Effects of L-Phenylalanine and Sodium Phenylpyruvate on the Formation of Adrenaline from L-Tyrosine in Adrenal Medulla *in vitro*

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While investigating the metabolism of L-tyrosine in rat-liver slices, Bickis, Kennedy & Quastel (1957) found that both L-phenylalanine and sodium phenylpyruvate inhibit the breakdown of this amino acid to acetoacetate and its incorporation into liver proteins. They suggested that these aromatic acids may affect other aspects of L-tyrosine metabolism, as, for example, the formation of hormones such as adrenaline, noradrenaline or thyroxine.

The biosynthetic pathway in bovine adrenal medulla has been established as: L-tyrosine $\rightarrow$ L-3:4-dihydroxyphenylalanine (dopa) $\rightarrow$ 3-hydroxytyramine $\rightarrow$ noradrenaline $\rightarrow$ adrenaline (Goodall & Kirshner, 1957). It is known that phenylpyruvate inhibits L-dopa decarboxylase in extracts of pig-kidney (Hartman, Akawie & Clark, 1955) and of guinea-pig adrenal medulla (Fellman, 1956), L-phenylalanine, however, having no effect. It has been suggested (Fellman, 1956) that interference with adrenaline formation by phenylalanine metabolites occurs by inhibition of L-dopa decarboxylase in adrenal medulla.

One of the phenomena associated with the inherited metabolic disorder, phenylketonuria, is a decreased concentration of plasma adrenaline. Testing 13 phenylketonuria patients and 117 normal humans, Weil-Malherbe (1955) found average basal adrenaline values of 0.62 and 1.52 $\mu\text{g./l.}$ respectively. Bickel, Gerrard & Hickmans (1954) reported that the blood concentration of tyrosine in phenylketonuria patients is normal or only slightly lower, but L-phenylalanine accumulates in the blood, reaching concentrations as high as 41 mg./100 ml. (2.5 mM) (Jervis, Block, Bolling & Kanze,

1940). This value is relatively constant in individual patients even after the ingestion of phenylalanine (Borek, Brecher, Jervis & Waelisch, 1950). Phenylpyruvic acid also accumulates in the blood; the concentration in fasting patients is usually not more than 1.8 mg./100 ml. (0.1 mM), but it may increase to four times this value after the ingestion of phenylalanine (Jervis, 1952). In normal human blood, the concentration of both L-tyrosine and L-phenylalanine is approx. 10 mg./100 ml. (0.06 mM) (Johnson & Bergeim, 1951) and phenylpyruvate is not detectable.

The present paper is concerned with the formation of adrenaline by adrenal medulla *in vitro* and the effects thereon of L-phenylalanine and phenylpyruvate under conditions approximating to the physiological. Although adrenal medulla contains ascorbic acid, additional quantities of this compound were added to the media in which the tissue was incubated in order to promote the conversion of tyrosine into dopa, considered by Holtz (1959) to be the rate-limiting reaction in the synthesis of catecholamines.

EXPERIMENTAL

Materials. Adult guinea pigs (800–1200 g.) were obtained locally. DL-[2- ^{14}C]Tyrosine (0.2 mc; 8.54 mg.) came from Atomic Energy of Canada Ltd. DL-[^{14}C]Adrenaline D-bitartrate (0.1 mc; 30.3 mg.) was obtained from Tracerlab Inc. L-Tyrosine, L-phenylalanine, sodium phenylpyruvate, DL-dopa, 3-hydroxytyramine-HCl, DL-noradrenaline and adrenaline (U.S.P.) were obtained from Nutritional Biochemicals Corp.

Preparation of biological material. Two guinea pigs were killed by decapitation and the adrenals were removed to chilled Ringer solution of the following composition: 0.14M-NaCl, 6 mM-KCl, 1 mM- CaCl_2 , 1 mM- MgSO_4 and 1 mM- KH_2PO_4 . The medullary tissue was prepared by the method of Hokin, Benfey & Hokin (1958). The bulk of the

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