PROFESSOR B. CHANCE

The Reactivity of Haemoproteins and Cytochromes

By B. CHANCE*

THE SECOND KEILIN MEMORIAL LECTURE

Delivered at a Meeting of The Biochemical Society on 5 July 1966 in the Chemistry Laboratory Lecture Theatre, University of Cambridge

This second Keilin Memorial Lecture marks several events that enhance our respect for, and appreciation of, the work of David Keilin on cytochrome, 41 years after the publication of his classical papers in *Nature* (Keilin, 1925a) and in the *Proceedings of the Royal Society* 'On Cytochrome, a Respiratory Pigment, Common to Animals, Yeast, and Higher Plants' (Keilin, 1925b). April this year marked the publication of Keilin's philosophy as set forth in his *History of Cell Respiration and Cytochrome* (Keilin, 1966). We are grateful to Joan Keilin, E. F. Hartree and E. C. Slater for their labours, which have made this work available to us, complete and full of the life and thoughts of this remarkable man. The content of a small colloquium in Philadelphia on haems and haemoproteins (Chance, Estabrook & Yonetani, 1966b) in April this year also followed the threads of Keilin's work at every turn, and emphasized their vitality.

Keilin's *History* reveals little explicitly of the character of the man, yet every page shows implicitly his honesty, keenness of observation and great scientific intuition. His interests went well beyond the existence of cytochrome; he was the first to observe the dynamics of cytochrome oxidation-reduction changes in a living animal, the wax moth (Keilin, 1925b). Through these observations he established the idea of the steady states of cytochrome: 'The absorption bands of *Galleria* after exertion or vibration of the wings, were never so strong as they appear in specimens exposed to pure nitrogen or to hydrogen cyanide' (Keilin, 1925b). The concept of the balanced steady states of oxidation and reduction in multi-enzyme systems (even now only partially understood) was one of Keilin's greatest intellectual contributions. It was one of his greatest personal excitements as well. He wrote: 'I must admit that this first visual perception of an intracellular respiratory process was one of the most impressive spectacles I have witnessed in the course of my work' (Keilin, 1966). He was perceptive enough to realize that the discovery of the existence of the enzyme was not sufficient; the demonstration

of its function *in situ* was essential. Thus it is indeed appropriate that a Keilin Memorial Lecture be set before a background of papers on multi-enzyme systems and their function, and that Professor Dixon should have organized a programme that represents one of Keilin's wide interests. For brevity and simplicity, however, I have selected for discussion only two aspects of multi-enzyme systems, electron-transport and respiratory-control mechanisms: the processes by which an electron leaves an electron carrier such as cytochrome *c*, and those by which the reactivity of a cytochrome such as *c* can be controlled to meet the needs of its biochemical function.

Electron transfer via cytochrome c

Structure and function of cytochrome c. Our focus on cytochrome *c* in this Lecture seems appropriate for several reasons. In his first paper (Keilin, 1925b) describing the visual spectroscopy of various tissues, particularly insect flight muscle, Keilin had already resolved MacMunn's four-banded spectrum into the α - and β -bands of three component haemochromogens, which he termed 'cytochromes'. One of them, cytochrome *c*, he was able to extract with water from powdered dried yeast. Later Keilin (1930) developed an extraction procedure yielding a more highly concentrated and purified yeast cytochrome *c*, which he employed for establishing the haemato-nature of the porphyrin (Hill & Keilin, 1930), for quantitative spectroscopic measurements (Dixon, Hill & Keilin, 1931) and for an estimation of the haematin iron content (Hill & Keilin, 1933). Highly pure heart cytochrome *c* was prepared by Theorell (1935, 1936) and by Keilin & Hartree (1937), and Theorell & Åkesson (1941) did pioneer work on its physical-chemical properties. As early as 1929, Keilin had proposed that cytochrome mediated the incompatible 'activated hydrogen' system of Weiland (1922) and Warburg's oxygen-activating iron-containing *Atmungsferment* (1924), a theory also proposed by the Japanese workers Shibata & Tamiya (1930). Warburg, Negelein & Haas (1933) proposed a sequential oxidation of Keilin's three

* Address: Johnson Research Foundation, University of Pennsylvania, Philadelphia 4, Pa., U.S.A.

cytochromes and the sequence of reaction was suggested by Ball (1938). Theorell (1935) proposed cytochrome *c* as the 'missing link' between *Atmungsferment* and the recently discovered pyridine nucleotide dehydrogenases and flavoproteins. These papers clearly established the haem nature of the molecule and the chemical structure of the haem, and laid the groundwork for a fuller description of its function as an essential carrier in electron transport and oxidative phosphorylation. This has been subsequently established by the work of many, including particularly Shibata (1935), Stotz, Altschul & Hogness (1938), Keilin & Hartree (1939), Marsh & Goddard (1939), Yakushiji & Okunuki (1940), Theorell (1944), Slater (1950a), Tsou (1952), Nielson & Lehninger (1954), Chance & Williams (1955a,b,c, 1956), Estabrook (1958), Smith & Conrad (1961) and King, Kuboyama & Takemori (1965).

The haem-protein linkages in cytochrome *c* were first explored by Theorell, who found that the porphyrin was anchored to cysteine residues by two thio ether bonds (Theorell, 1938, 1939) and proposed that two histidine imidazole groups are bound to the iron (Theorell & Åkesson, 1941). Somewhat later, Theorell (1956) studied the major peptides and proposed a molecular structure for cytochrome *c* in the region of the haem. More recent work, particularly that of Margoliash (1966) and Margoliash & Schejter (1966), on the constancy of certain portions of the peptide sequences in cytochrome *c* from different species, confirms Theorell's views but also raises the possibility that the two haem linkages are to histidine-18 and methionine-80. This observation is supported by Harbury, Myer, Murphy & Vinogradov (1966) in their studies of a *Pseudomonas* cytochrome *c* that contains only one histidine residue and in which the methionine residue identifies itself as a haem linkage by the loss of the low-spin spectrum on selective carboxymethylation. Although the exact nature of the haem linkage of cytochrome *c* may be undecided, the solution of the problem of the ferric form by X-ray crystallography (Dickerson, Kopka, Weinzierl, Varnum & Borders, 1966) will surely settle this point. While we wait expectantly for the X-ray crystallographic results, certainly some of the chemical data seem reasonably secure. For example it now seems beyond doubt that the haem of cytochrome *c* is well buried within its protein structure (Theorell & Åkesson, 1941), and the interpretation of amino acid sequences (Margoliash & Schejter, 1966) adds further support to this view. In fact, Theorell's 'haem in the crevice' idea implies that electrons be conducted from the haematin iron via its imidazole or methionine linkages to the electron acceptor, as described in detail by him (Theorell, 1944), and suggests an active participation of these other groups of the haem in the electron-

entering and -leaving reactions that represent that physiological role of cytochrome *c*.

Electron-donating properties of cytochrome c. These properties have been studied in solution by a variety of methods. Perhaps the simplest determinations are made when cytochrome *c* is its own oxidant and reductant, and the effects are observed by shifts in the nuclear-magnetic-resonance spectra (Kowalsky & Cohn, 1964). The second-order constant for this reaction is rather small, $10^4 \text{ M}^{-1} \text{ sec}^{-1}$ (Kowalsky, 1966). A very much larger velocity constant, about $10^7 \text{ M}^{-1} \text{ sec}^{-1}$, was computed from the kinetic and steady-state properties of the respiratory chain (Chance, 1952) and was directly obtained by Smith (1954) with purified cytochrome oxidase as oxidant. The extraordinary electron-donating activity of cytochrome *c* becomes apparent in its reaction with the peroxidase-peroxide intermediate described by Keilin & Mann (1937), particularly in the yeast enzyme (Altschul, Abrams & Hogness, 1940). Our preliminary estimates for this constant reached $10^8 \text{ M}^{-1} \text{ sec}^{-1}$ (Chance, 1951b; Beeststone, 1960), and the present value for Yonetani's pure crystalline enzyme is $5 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ (Yonetani & Ray, 1966), a value which exceeds that for the reaction of small ligands with haemoproteins, including the reactions of oxygen with cytochrome oxidase (Chance & Schindler, 1965) or hydrogen peroxide with peroxidase (Chance, 1951a; Ludwig & Kuby, 1955; Gibson & Greenwood, 1963). The reaction of ferrocytochrome *c* with the enzyme-substrate complex of yeast cytochrome *c* peroxidase is the most rapid protein-protein interaction that has yet been measured in solution, and closely approaches the limits set by collisions of the two proteins.

First-order velocity constants can indicate a maximum value for the residence time of cytochrome *c* in contact with its electron acceptor, and may ultimately afford some insight into the actual time required for the electron to leave the iron and move through the methionine and histidine towards its ultimate electron acceptor. The first-order velocity constant of the reaction is 10^4 sec^{-1} (Scheme 1) and the half-time for cytochrome *c* oxidation by the peroxidase complex is $70 \mu\text{sec}$.

This is a remarkably short time, in view of the fact that it is computed for an overall turnover number of enzymic activity, and includes the time for the cytochrome *c* molecule to diffuse to the surface of the enzyme-substrate intermediate, donate its electron to the peroxide intermediate and diffuse from the site to allow the next molecule to approach. Two factors are involved in determining this turnover time: the actual diffusion time and the actual electron-transfer time. The latter may be much less than $70 \mu\text{sec}$.

Yonetani's recent data on the mechanism of

Scheme 1. Mechanism of reaction of ferrocycytochrome *c*, cytochrome *c* peroxidase and H_2O_2 . The overall stoichiometry is given in the top line and the sequential steps involve the reaction of enzyme (*E*) with a substituted or unsubstituted peroxide ($RO \cdot OH$) at a velocity constant between 10^7 and $10^8 M^{-1} sec^{-1}$ to form the red enzyme-substrate compound, 'complex *ES*', which retains the 2 oxidizing equivalents of the peroxide. The remainder of the cycle indicates sequential reactions with cytochrome *c*, involving postulated ternary intermediates of 2 ($ES-c''$) or 1 (ES^*-c'') oxidizing equivalents (Yonetani & Ray, 1966).

interaction of yeast cytochrome *c* peroxidase with cytochrome *c* are of great interest. Though the combination of the peroxidase with hydrogen peroxide forms the typical red intermediate first observed with horseradish peroxidase by Keilin & Mann (1937), the reaction differs from that of the horseradish enzyme in the sense that no green precursor (Theorell, 1941) of the red intermediate can be observed, even at 1 msec. after adding hydrogen peroxide (Yonetani, Schleyer, Chance & Ehrenberg, 1966). Thus cytochrome *c* peroxidase reacts with hydrogen peroxide by a mechanism similar to that put forward by Keilin & Mann (1937) and studied by us in detail (Chance, 1943), rather than that proposed by George (1956) on the basis of stoichiometric studies of the yeast and horseradish enzymes. The red intermediate formed by the yeast enzyme retains both oxidizing equivalents of peroxide, and for this reason Yonetani terms it 'complex *ES*'. It is possible that one of the oxidizing equivalents is associated with the haematin iron and the other with the protein in the form of a free radical (Yonetani *et al.* 1966). If this free radical were nearer the surface of the protein, it could act as an electron-transfer mediator between cytochrome *c* and an inaccessible oxidizing equivalent of the iron atom (see also Nicholls, 1964).

Temperature-independent electron transfer. To

investigate further the reaction mechanism for the oxidation of cytochrome *c*, we studied the speed of the light-induced oxidation of cytochrome in photosynthetic bacteria (Duysens, 1954; Chance & Smith, 1955; Olson & Chance, 1960), for the following reasons. First, we have found that electron-transfer mechanisms in bacteria have a simpler form than in mammalian systems. Secondly, we have studied whole-cell suspensions of living organisms since the rate of cytochrome *c* oxidation may be slowed by formation of subcellular particles (Chance *et al.* 1965; see also below, and Keilin & Hartree, 1940, 1949). Thirdly, we have been able here to employ the 'instant oxidant' provided by light-activated bacterial chlorophyll.

In intact cells of the purple sulphur bacterium *Chromatium* D, the 'primary process' of electron transfer between cytochrome *c* and activated chlorophyll can be observed even under conditions where diffusion and thermal motion of cytochrome *c* are 'damped out' at extremely low temperatures. [Other optical changes related to chlorophyll have been observed, particularly in chromatophores (Clayton, 1962; Parson, 1966). Though we have as yet been unable to show that such changes are essential to cytochrome oxidation, we would include these intermediates as a component of activated chlorophyll in Schemes 2 and 3.] Nishimura and I have found that cytochrome oxidation caused by steady illumination of bacterial chlorophyll is insensitive to temperature in *Chromatium*; we observed cytochrome *c* oxidation in a few tenths of a second after illumination of the frozen bacteria at $77^\circ K$ (Chance & Nishimura, 1960). We attributed this reaction to a cytochrome $c_{423.5}$ (the subscript refers to the position of the peak of the Soret band of the reduced cytochrome), which has recently been isolated by Bartsch (1966) as a typical cytochrome of type *c*, but of a particulate nature and with a redox potential of approx. 0 v. Vredenberg & Duysens (1964) disputed the speed of the low-temperature reaction; however, L. N. M. Duysens (personal communication) has since stated that his observations were in error owing to the slow response of his recording instrument. The matter has been resolved by our development, over the last 3 years, of a new technique (Fig. 1). An extremely short light-pulse is obtained by laser action, whereby a flash from a surrounding xenon tube populates the excited state in a chromium-supplemented ruby crystal (Townes & Schawlow, 1958). Light-emission in the deactivation of this excited state stimulates the emission of other atoms and leads to a series of brilliant monochromatic flashes at $694 m\mu$ for about $500 \mu sec$. To concentrate these into a single giant flash of $20 m\mu sec$ duration, a rotating-mirror arrangement prevents the laser action from starting until all the chromium atoms have been excited by

Fig. 1. Simplified diagram indicating the essential elements of the 'Q-switched' laser and fast spectrophotometer. The wavelength of the laser excitation light is $694\text{ m}\mu$ and that of the measuring light is $422\text{ m}\mu$. The laser excitation proceeds from the left-hand side of the diagram and is reflected on the sample of bacteria in the cuvette by a mirror. The measuring light impinges directly on the cuvette, and is obtained from a grating monochromator provided with a shutter and a tungsten lamp. In the actual operation, the monochromator lamp is pulsed to a high intensity a short time before the laser flash by a special electronic circuit. For complete details, see DeVault (1964).

the xenon flash lamp in a process termed 'Q-switching'. By suitable intensity adjustments, enough photons fall on the cells to activate a number of chlorophyll molecules equal to the number of cytochrome *c* molecules in the suspension of bacteria (see also Chance, Schleyer & Legallais, 1963).

The oxidation of reduced cytochrome *c* is measured by the disappearance of the Soret band. For this purpose we have employed a fast spectrophotometer devised largely by DeVault (1964), in which the light-source for the monochromator is at maximal intensity 10–20 msec. before the laser flash. This minimizes the problems that arise when light for spectrophotometric measurement exposes the sample to a pre-illumination that may modify the flash-activated absorption changes (Witt *et al.* 1965).

In Fig. 2, the time-sequence of events is shown as an oscilloscope trace for a sample of *Chromatium* at 85°K . The activation of chlorophyll is complete in $30\text{ m}\mu\text{sec.}$, and the extinction decrease due to cytochrome oxidation follows exponential kinetics with a half-time of 2 msec. As yet there is no evidence for a prior fast step, involving another cytochrome or a component of chlorophyll. The cytochrome is identified by its difference spectrum as cytochrome $c_{423.5}$ (Chance & Nishimura, 1960), the component characterized by Olson & Chance (1960).

Thus we may write simplified equations for the

Fig. 2. Kinetics of cytochrome oxidation at 85°K after a $30\text{ m}\mu\text{sec.}$ laser flash. The trace shows an upward deflexion corresponding to the oxidation of cytochrome $c_{423.5}$. The chlorophyll concentration is $\sim 0.3\text{ mM}$ and the optical path is 2 mm.

physical and chemical processes involved in the reaction of bacterial chlorophyll (BChl) with light quanta ($h\nu$) to form activated chlorophyll (BChl^*), which serves as the oxidant for ferrocytochrome $c_{423.5}$:

The fate of the electron on chlorophyll (BChl^-) has not yet been determined by kinetic studies.

Fig. 3. Temperature-independence of cytochrome oxidation at low temperatures in frozen suspensions of *Chromatium* D obtained with a 30 μ sec. flash, the squares with a 500 μ sec. flash. Other conditions were as described in Fig. 2. (See DeVault & Chance, 1966b.)

Fig. 3 illustrates the effect of temperature on the rate of cytochrome *c* oxidation in the range 34–120° K. A hydrogen-liquefying device gives continuous temperature variation in this region. The graph shows no temperature-dependence of the reaction velocity in excess of that corresponding to an apparent energy of activation of 80 cal./mole; in fact, the data predict a 2 msec. half-time at 0° K. Thus we find a temperature-insensitive cytochrome *c* oxidation after illumination of the frozen bacteria (DeVault & Chance, 1966a; Chance, DeVault, Hildreth, Parson & Nishimura, 1966a; Chance & Nishimura, 1960). Although *Chromatium* D is the only organism in which this reaction has so far been detected, these studies afford detailed evidence that cytochrome *c* is capable of an electron-transport process that does not require thermal motions of the cytochrome *c* molecule relative to the molecules with which it reacts, or any relative motions of the constituents of the cytochrome *c* molecule itself. In fact, this finding seems to favour electron transfer over hydrogen transfer in cytochrome *c*.

Reaction mechanisms at low temperatures. The finding that cytochrome *c* can lose an electron in a half-time of 2 msec. at 34° K seems to rule out bond-breaking or conformation changes as essential to its electron-transport mechanism. Thus we may regard the postulated conformation changes of cytochrome *c* as secondary consequences, rather than causes, of the electron-transport reaction. For example, Matsubara, Ando & Okunuki (1965) find different extents of carboxymethylation of the oxidized and reduced forms. A number of investigators employing optical rotatory dispersion at low and high magnetic fields attribute the distinctive differences between the oxidized and reduced forms to changes of protein structure (Harbury *et al.* 1966; Shashoua & Estabrook, 1966). Bond-breaking, as required by the switch of the sixth ligand of the iron from one imidazole to another to serve as an

Scheme 2. Schematic drawing of the relationship of cytochrome *c* and bacterial chlorophyll, separated by a barrier through which electron tunnelling may occur. The dimensions of the barrier are taken to be 30 Å in width and 1 ev in potential.

'imidazole pump' for electrons (Urry & Eyring, 1965a,b), or from histidylimidazole to methionine (Hettinger, Langer, Vinogradov & Harbury, 1966), seems unlikely. However, we may consider the possibility that a change of haem linkage or conformation may determine whether or not cytochrome *c* of one type or another can be an effective electron donor at low temperatures.

The remarkable feature of the low-temperature reaction is that it is slow, and the simple electron-conductivity mechanism, such as that proposed initially by Evans & Gergeley (1949) and from time to time since then, would be expected to give very short electron-transfer times (less than 0.1 μ sec.) for electron conduction from the iron through its haem linkage to the electron acceptor (Scheme 2). The processes that occur in chlorophyll during the 30 μ sec. light-flash are very rapid: photon capture, followed by a singlet-triplet state conversion or

formation of a metastable state that would act as an electron acceptor for cytochrome *c*, should take only 0.01–1 μsec . We may attribute the slowness of the electron-transfer process to a barrier between cytochrome *c* and chlorophyll, and consider the long half-time for the oxidation of cytochrome *c* to be a measure of the probability with which electrons may penetrate this barrier. If, for example, electrons of a potential of less than 1 v impinge on a barrier 1 v high, classical mechanics would predict that no electrons would pass. The quantum-mechanical approach, however, indicates a finite probability that, for a barrier of finite width, electrons of energy less than the barrier height would successfully penetrate it. The mechanism for this is termed 'quantum-mechanical electron tunnelling', the name implying that some electrons will 'tunnel' through the barrier instead of going over it. For the case where the energy levels of the electrons on either side of the barrier are approximately equal, the probability of such electron flow may be expressed in the following equation for a rectangular barrier:

$$t_{\frac{1}{2}} = \frac{0.7}{16f} \left(2 + \frac{V-E}{E} + \frac{E}{V-E} \right) \exp \left\{ -\frac{a}{\hbar} [8M(V-E)]^{\frac{1}{2}} \right\} \quad (3)$$

where f is the frequency of collision of the electron with the barrier, V is the height of the barrier in eV; E is the electron energy, $\hbar$ is Planck's constant/ 2π ; M is the electron mass and a is barrier width.

One essential feature of eqn. (3) is that it does not contain a temperature-dependent parameter, and in this respect is one of the few reaction mechanisms to meet the needs of the experimental data (f , the frequency factor, is taken to arise from electron conductivity from the iron of cytochrome *c* to the barrier, and in this sense is also a temperature-independent parameter). In the dark, electron tunnelling is very improbable because the energy levels in chlorophyll are not equal to those in cytochrome *c*. On illumination of chlorophyll, however, its energy levels approach those of cytochrome *c* and electron tunnelling becomes possible.

Eqn. (3) may be used to calculate the half-time for tunnelling such a rectangular barrier, for purposes of comparison with the experimentally determined half-time for cytochrome *c* oxidation. Though it is obvious that there is a wide range of variables giving a half-time of 2 msec., a particular set that appears consistent with the physical properties of cytochrome and chlorophyll involves a barrier width, a , of 30 Å, an electron energy, E , of 1 v, and a frequency factor, f , of 10^{15}sec^{-1} . The barrier height is taken to be 2 v, twice the value of the electron energy E . The value of the constant $\hbar$ is 1.05×10^{-34} joule-sec. A complete discussion of

this equation, and the possible parameter ranges for it, is given elsewhere (DeVault & Chance, 1966b).

The barrier has not as yet been identified in chemical terms, but remains a physical concept at present. However, it will become apparent in the discussion that follows (see Schemes 3 and 4, for example) that electron tunnelling through the protein moiety of cytochrome *c* seems to be inconsistent with the proposed mechanism. For this reason, we suggest that the tunnelling barrier is composed of lipid. However, at the present time, we can attach quantitative values only to the physical, and not to the chemical, parameters of the electron-tunnelling equation.

Temperature-dependent electron transport. Fig. 4 is a typical Arrhenius plot, which indicates that, above 120°K, a component of the electron-transport rate increases with increasing temperature, with an apparent energy of activation of 3300 cal./mole. At room temperature, the reaction proceeds 1000 times faster than at 77°K, and the half-time shortens to 2 μsec . [The 18–20 μsec . half-time for cytochrome *c* oxidation in *Chromatium* at 299°K reported previously (Chance & DeVault, 1964; Chance, Schoener & DeVault, 1965) has not been reproduced.]

Fig. 4. Temperature-dependent oxidation of cytochrome *c* in *Chromatium*. Ordinates are proportional to the logarithms of the half-times; abscissae are proportional to the reciprocals of the absolute temperatures given on the lower scale (DeVault & Chance, 1966b).

Scheme 3. Schematic diagram indicating mechanisms of electron transport between two different cytochromes and bacterial chlorophyll, one involving a tunnelling barrier and operative at low temperatures, the other involving a reaction requiring thermal energy and a very narrow tunnelling barrier.

Scheme 3 illustrates possible mechanisms for the more rapid reaction at higher temperatures. One explanation (left-hand side) involves only cytochrome $c_{423.5}$ and suggests that thermal-agitation energy now allows this cytochrome to achieve a more favourable orientation with regard to the active centre of the electron acceptor, thus altering the properties of the quantum-mechanical barrier between the two. Though this is a feasible mechanism from the physical standpoint, chemical data imply that a second cytochrome is involved. Oxygenation of the bacterial suspension in the dark at room temperature causes oxidation of the cytochrome $c_{423.5}$, obliterating its response to chlorophyll illumination. [We find that the low-temperature response is also slowed by repeated freezing and thawing of the cells or by formation of chromatophores, and thus involves fragile structures or intimate relationships of chlorophyll and cytochrome (see also Vredenberg & Duysens, 1964; Morita, Edwards & Gibson, 1965).] Therefore an alternative explanation attributes the temperature-dependent reaction to the non-autoxidizable cytochrome c_{422} (Olson & Chance, 1960; Vredenberg & Duysens, 1964). The complete temperature profile may be due to the merging of the reaction times of two cytochromes absorbing at very nearly the same wavelength in the Soret region. [Unpublished experimental data (D. DeVault) indicate biphasic reaction kinetics at 140°K , due to the separate oxidation of cytochromes of c_{422} and $c_{423.5}$. At room temperature there is additional evidence for sequential reactions: after the $2\mu\text{sec.}$ oxidation, there is a further cytochrome change with a half-time of approx. $50\mu\text{sec.}$ (see Chance *et al.* 1966a; Morita *et al.* 1965).]

The second type of reaction is shown on the right-hand side of Scheme 3. Cytochrome $c_{423.5}$ has its

haem so orientated that electron transfer can occur by tunnelling through the barrier between it and the electron acceptor. The haem of cytochrome c_{422} , however, is so orientated that electron transfer by the tunnelling mechanism cannot occur, and thermal activation is required to reorientate the haem appropriately. When thermal agitations are adequately frequent, cytochrome c_{422} becomes the preferred electron donor. This mechanism assumes that at temperatures below 120°K cytochrome c_{422} 'freezes' in a position that does not permit electron tunnelling, whereas cytochrome $c_{423.5}$ 'freezes' in a position that does permit tunnelling. Obviously, other arrangements are possible, especially if another electron carrier is interposed between cytochrome and chlorophyll.

A third mechanism that may be possible at higher temperatures is shown in the centre of Scheme 3 and involves cytochrome-cytochrome interaction of the type discussed below for the respiratory chain (see Scheme 4). In this case, at temperatures above 120° , thermal activation could cause collisions of cytochrome c_{422} with cytochrome $c_{423.5}$, resulting in a more rapid and temperature-dependent oxidation of the latter.

In summary, our studies provide experimental evidence for four possible steps in electron donation from cytochrome c to chlorophyll: first, an unmeasurably rapid electron conduction from the iron through its ligands to some point on the periphery of the protein; secondly, a slow, and therefore fairly improbable, temperature-independent electron-tunnelling reaction with a half-time of 2msec. through the barrier between cytochrome $c_{423.5}$ and its electron acceptor; thirdly, when energy for thermal agitation is available, a temperature-sensitive reaction with an apparent energy of activation of 3300 cal./mole and a half-time of only $2\mu\text{sec.}$ at room temperature, occurring between a second cytochrome (c_{422}) and bacterial chlorophyll; fourthly, an intercytochrome electron transfer between cytochromes c_{422} and $c_{423.5}$ also requiring thermal energy.

Light-induced cytochrome f oxidation. The pioneer work of Robert Hill on cytochrome f of green plants (Hill & Bhagvat, 1939) and subsequent studies on the light-induced oxidation of this component (Duysens, Ames & Kamp, 1961; Lundegårdh, 1961; Witt, Müller & Rumberg, 1963) have led us to attempt to measure its oxidation time. Again, we have found the most effective material to be the living cell, in this case *Porphyridium*. According to recent experiments in our Laboratory by M. Nishimura (Table 1), cytochrome f shows an oxidation time of $14\mu\text{sec.}$, somewhat slower than that of *Chromatium* but within the same time range. This reaction appears to be temperature-sensitive, fitting into the third category above.

Table 1. *Oxidation times for cytochromes of type c in biological reactions*

Cytochrome type ...	...	c	c _{low temp.} * (< 120°K)	c _{high temp.} † (> 120°K)	f	c
Source	Beef		<i>Chromatium</i>	<i>Chromatium</i>	<i>Porphyridium</i>	Liver
State	Soluble		Cells	Cells	Cells	Mitochondria
Electron acceptor	Cytochrome c peroxidase		Bacterial chlorophyll	Bacterial chlorophyll	Chlorophyll a	Cytochrome a
Oxidant	H ₂ O ₂		Radiation	Radiation	Radiation	O ₂
k (sec. ⁻¹)	1.4 × 10 ⁴		350	3 × 10 ⁵	5 × 10 ⁴	380
t _{1/2} (msec.)	0.05		2	0.002	0.014	1.7
Temp. (°K)	298		34–120	298	298	306

* Probably cytochrome c_{423.5}.† Probably cytochrome c₄₂₂.*Electron transport in mitochondria and chloroplasts*

Interaction of cytochromes c and a. The studies described above afford a basis for the interpretation of experimental data on electron transport between cytochromes c and a in animal mitochondria. Here we have sought a substitute for the light-pulse that we employed as an 'instant oxidant' in the photosynthetic system. We find that cytochromes a and a₃ accept electrons from cytochrome c sufficiently slowly for cytochrome c oxidation to be rate-limiting in the following reaction sequence:

Improvements to the Hartridge-Roughton method (Roughton & Chance, 1961) for rapidly mixing oxygen with reduced cytochrome oxidase allow the rapid formation of an electron acceptor for cytochrome c. Experimental results obtained with the regenerative-flow apparatus are shown in Fig. 5 (Chance, 1964). Here we have plotted, as a function of time, the extent of oxidation of cytochrome c of rat-liver mitochondria; the curve represents an approximately first-order relationship, giving a half-time of 1.8 msec. and a value for k₅ of 380 sec.⁻¹. The reaction is temperature-sensitive (Chance *et al.* 1965).

To determine whether the reaction of cytochrome oxidase with oxygen was rate-limiting under these conditions, two sets of data were obtained, one at 12 μM- and the other at 48 μM-oxygen. It is apparent that the points from the two concentrations coincide closely. We have measured the oxidation time for cytochrome a as well (k₃) and found it to be approximately twice as rapid as that (k₅) for cytochrome c (Chance *et al.* 1965). Therefore the reaction of cytochrome oxidase with oxygen under these experimental conditions appears to fulfil the

Fig. 5. Rapid-flow measurements of the extent of reaction (ordinate) against time after mixing (abscissa) for the oxidation of cytochrome c of rat-liver mitochondria, after reaction of cytochrome oxidase with molecular oxygen at 12 μM (○) or 48 μM (□) concentration. The half-time of the reaction is 1.8 msec. (k 380 sec.⁻¹). The rat-liver mitochondria, suspended in a medium containing mannitol (0.275 M) and sucrose (0.075 M), are present in a concentration of 6.6 mg./ml., and are supplemented with succinate (5 mM) as substrate. The temperature of the reaction medium was 32°. Experiments were carried out in the regenerative-flow apparatus described by Chance (1964).

requirements for an 'instant electron acceptor' for cytochrome c, and we may presume that we are measuring the capability of cytochrome c for donating electrons in the respiratory chain of the intact, phosphorylating mitochondria.

Electron transfer from cytochrome c to cytochrome a is remarkably slow when compared with the transfer rates in the reactions of cytochrome c with peroxidase and peroxide or in *Chromatium* at 298°K, and with the rates in the reactions of cyto-

chrome *f* (see Table 1). Keilin & Hartree foresaw the important biological principles that are brought forward here. Observing the fall in catalytic activity of electron-transport systems that results from cellular disruption, they proposed that the efficiency of such systems in living cells is due to optimum conditions of mutual accessibility of the catalytic components (Keilin & Hartree, 1940, 1949). We can now extend this concept by stressing that a hindrance to mutual accessibility can be 'built into' the mitochondrion or the whole cell as a control mechanism.

One possibility for control that we have already considered is the barrier to electron tunnelling shown in Scheme 2. A second possibility is that control may be based on the requirement for a certain amplitude of thermal motion as illustrated in Scheme 3 for the photosynthetic system and in Scheme 4 for the respiratory chain. The latter Scheme emphasizes that electron transfer may involve, not just motion of the haem of cytochrome *c* relative to a fixed point in the molecule (Scheme 4A), but also a juxtaposition of the haems of both cytochromes *a* and *c*, which considerably lowers the probability of a reaction between them (Scheme 4B). At the moment of adding oxygen to the reduced carriers of the mammalian respiratory chain, some haems will be found in a proximal orientation so that electron transfer between cytochromes *c* and *a* will occur extremely rapidly (Scheme 4A). However, a distal orientation is also possible (Scheme 4B) in which a rotation of 180° or an equivalent translation would be necessary to bring the haem of the electron-bearing cytochrome and its acceptor into adequately close contact. In this case, the large ratio of the reaction rates in the chlorophyll–cytochrome system to those in the cytochrome *a*–*c* system is consistent with the need for a greater degree of rotation or translation in one than in the other.

Controlled electron transport and energy conservation. It is only at this point that we go beyond the

range of Keilin's experiments to consider the mechanism by which electron-transfer energy is coupled to the phosphorylation of ADP, the movement of cations and the reversal of electron transport in portions of the respiratory chain. Here we must emphasize the distinction between the slower, energy-conserving, reactions of steady-state oxidative phosphorylation and transient electron-transfer reactions at maximal rates of electron flow. In energy-conserving reactions, electron flow proceeds at a rate controlled to meet the needs of energy-linked reactions, an extension of the view proposed by Lardy (1955) and amplified by Slater (1955c) and ourselves (Chance, 1955).

In the generalized symbols employed by Slater (1953):

or in the more specific terminology of Chance & Williams (1956) for a particular site involving cytochromes *c*, *c*₁ and *a*:

the substances, C or I, serve to protect the components A or *c''* from further reaction until the energy denoted by '~' is transferred to some suitable acceptor by any of the processes mentioned above. If, for example, the electron-transfer reactions involving either the oxidation or reduction of cytochrome *c* occur more rapidly than the binding of *c* and I, then electron transport would be uncontrolled and energy conservation ineffective. Though we do not know the rates of the energy-conserving reactions relative to those of the electron-transfer process, a comparison between the maximal rates of electron transport coupled to phosphorylation and those of transient electron flow may be useful.

Obviously, electron transport without a tunnelling barrier is too fast (half-times of 1 μsec.) for effective energy conservation, and even the 2–14 μsec. half-times observed for cytochromes *c*

Scheme 4. Schematic diagram indicating two types of electron transfer between cytochromes. Scheme 4(A) represents cytochromes *c* and *a* in a proximal orientation allowing immediate electron transfer. Scheme 4(B) indicates the haems of cytochromes *c* and *a* to be in a distal orientation that requires rotation for electron transfer to occur.

and *f* in photosynthetic bacteria at 298°K are probably too short. One may, then, question the function of the rapid reaction between cytochrome and chlorophyll. One answer is that the energized state of chlorophyll is metastable, and energy must be transferred to cytochrome before competing processes intervene. In this case, the cytochromes may serve the purpose of moving the electron acceptor away from the active centre of chlorophyll to a point where slower electron transfer by a collision process may take place, thereby rendering the electron-transfer process accessible to energy-conserving reactions. This viewpoint suggests that, though the primary reaction of cytochrome and chlorophyll may not be directly involved in energy conservation, some rate control at an energy-conserving step elsewhere may be required.

Controlled electron transport in chloroplasts. In the two sequences of electron transport that involve energy conservation, photo-phosphorylation and oxidative phosphorylation, we may look for sites of energy conservation by observing the effects of ADP and uncouplers on the oxidation-reduction states of cytochromes *c* or *f*. These responses may be evaluated according to the cross-over theorem (Chance & Williams, 1956), which allows the identification of interaction sites in a linear multi-enzyme sequence. Between the initial electron donor and the ultimate electron acceptor of a given reaction sequence, there may be cross-over points at which the steady state shifts from an oxidation to a reduction, with an increase of electron flow through the system. Photosynthetic electron transfer is uniquely suited to cross-over study, since it can be activated either by mainly oxidizing equivalents on illumination with light at 710mμ (system I) or by reducing equivalents on illumination with light at 640mμ (system II) (Duysens *et al.* 1961). However, the systems overlap somewhat and considerable activation of system I also occurs with 640mμ illumination. One representation of photosynthetic electron transport might be:

in which ChlY represents the system II chlorophyll, PQ is plastoquinone, *b* is cytochrome *b*, PC is plastocyanine, *f* is cytochrome *f*, PchlX is the system I chlorophyll, FD is ferredoxin and Fp is flavoprotein (for further discussion see Duysens, 1966). Thus two chlorophyll components are involved in the photosynthetic sequence, with cytochrome *f* and a number of other electron-transport components interposed between them in a sequence that is not yet clearly defined (broken arrows). The system I chlorophyll *a* is that responsible for the rapid oxidation of cytochrome *f* in *Porphyridium*, and is

Fig. 6. Comparison of the responses of cytochrome *f* in spinach chloroplasts to ADP and to an uncoupling agent (NH_4Cl) with the responses of cytochrome *c* of rat-liver mitochondria to ADP and Ca^{2+} addition, and to an uncoupling agent (dicoumarol). Both experiments were carried out with a double-beam spectrophotometer. In Fig. 6(A), 96 μg. of chlorophyll/ml. is present in a 15 mM-tris-HCl buffer, pH 7.8 at 25°. The intensity of red illumination (640 mμ) is about 10 mμeinsteins $\text{cm}^{-2} \text{sec}^{-1}$, and is obtained through an interference filter. The concentrations of the other reagents are as indicated. Diquat is a derivative of bipyridyl, and serves as a low-potential electron acceptor. In Fig. 6(B) is shown a suspension of rat-liver mitochondria (4.0 mg. of protein/ml.) suspended in a medium made iso-osmotic with mannitol (0.225 M) and sucrose (0.075 M), and including MgCl_2 (5 mM), sodium potassium phosphate (3 mM) and succinate (3 mM). The concentrations of ADP, Ca^{2+} and dicoumarol are as indicated. Experiments measured in the double-beam spectrophotometer with wavelengths set at the values indicated, and with light-absorption sensitivity indicated on the scale at the right-hand side of each diagram. The time-scale of each diagram is also included. Fig. 6(A) is based on unpublished work by M. Avron & B. Chance; Fig. 6(B) is based on unpublished work by B. Chance & B. Schoener.

analogous to the bacterial chlorophyll (BChl) responsible for cytochrome *c* oxidation in the purple bacterium *Chromatium* (see eqns. 1 and 2, Table 1 and Scheme 3).

For Fig. 6 we have employed the dual-wavelength spectrophotometric technique to measure the steady-state oxidation-reduction levels of cytochrome *f* in suspensions of chloroplasts (Avron & Chance, 1966) and to record its response to illumination or to the addition of ADP or uncouplers. In these experiments, continuous rather than pulsed (laser) illumination is employed in order to establish

steady-state electron flow. Light at $640\text{ m}\mu$ activates both system I and system II. Starting at the left-hand side, an upward deflexion of the trace corresponding to decreased extinction at $554\text{ m}\mu$ indicates an oxidation of cytochrome *f* caused by $640\text{ m}\mu$ illumination. There is a further oxidation after the addition of the electron-acceptor diquat (1,1-ethylene-2,2'-bipyridolium dibromide; Zweig, Shavit & Avron, 1966). On addition of ADP to activate electron transport and phosphorylation, or of ammonium chloride to uncouple electron transport, however, a downward deflexion, indicating a reduction of cytochrome *f*, is recorded. No evidence of cytochrome oxidation on activation of electron transport is observed. [In laser experiments, the $694\text{ m}\mu$ illumination occurs so rapidly that system I responses predominate, and uncoupling agents cause no acceleration of the rate of cytochrome *f* oxidation (Hildreth, 1966).] Thus there appears to be no evidence for a cross-over point or an energy-conservation site between cytochrome *f* and chlorophyll in chloroplasts; instead, the energy-conservation site appears to be between the electron-donating chlorophyll component (system II) and cytochrome *f* (Scheme 4), where slower reactions are involved and possibilities for an energy-conserving interaction become more likely. A similar situation seems to exist in the photosynthetic bacteria, where no evidence is at present available for a photo-phosphorylation site between cytochromes $c_{423.5}-c_{422}$ and chlorophyll; studies with inhibitors and uncouplers point to phosphorylation on the electron-accepting side of these components (Baltscheffsky & Arwidsson, 1962).

Energy-conservation sites in mitochondria. Though phosphorylation sites in mitochondria are often identified by the use of selective substrates (Neilson & Lehninger, 1954; Maley & Lardy, 1954; Slater, 1950b, 1955b), the cross-over theorem identifies more precisely an energy-conservation site between cytochromes *c* and *a* (Chance & Williams, 1956). Fig. 6(B) illustrates the response of cytochrome *c* of rat-liver mitochondria to various activators of electron flow. Here we plot as a function of time (left to right) the changes in the steady-state levels of cytochrome *c* ($E_{550}-E_{540}$) caused by addition of $170\text{ }\mu\text{M}$ -ADP, $300\text{ }\mu\text{M}$ - Ca^{2+} and $50\text{ }\mu\text{M}$ -dicoumarol. Adequate time is allowed for the reactions with ADP and Ca^{2+} to go to completion, and thus cyclic responses are obtained. All three reagents stimulate electron transport and all three cause a decreased extinction at $550\text{ m}\mu$, corresponding to an oxidation of cytochrome *c*. The degree to which the reagents affect the oxidation-reduction levels of cytochrome *c* depends on their activity in stimulating electron transport.

On addition of ADP, the oxidation of cytochrome

c may be accompanied by the reduction of cytochrome *a*, defining a cross-over point at which electron flow is controlled and energy-coupling occurs. We may now consider whether electron-transfer times in steady-state oxidative phosphorylation are as short as the transient electron-transport times observed on the addition of oxygen to the reduced carriers.

The transient oxidation rate for cytochrome *c* corresponds to a turnover number of 380 sec^{-1} , and values exceeding this have been observed for cytochromes *a* and a_3 (Chance *et al.* 1965). Steady-state rates of electron transport do not approach these values: in rat-liver mitochondria at 25° , 5 sec^{-1} is an appropriate value for the active, ADP supplemented, state (state 3) and 0.5 sec^{-1} for the resting, ADP-deficient, state (state 4). In other words, the energy-conserving system does not function rapidly and may not couple efficiently at maximal rates of electron transport. It is noteworthy that turnover numbers for cytochrome *f* in steady-state photosynthesis ($10\text{--}20\text{ sec}^{-1}$) are also small compared with the maximum electron-transfer rate capability.

Table 2 summarizes data on the state 3 and state 4 oxidation-reduction levels of cytochrome *c* in a variety of materials. It is apparent that cytochrome *c* is between 8 and 35% reduced in state 4, in spite of the fact that the oxidation rate greatly exceeds the turnover number of this component. Further, when the electron-flow rate increases five- to ten-fold in state 3, cytochrome *c* may become more reduced, or more oxidized (as in the locust flight-muscle sarcosomes). In terminal inhibition of the respiratory chain by azide, a much larger increase of reduction occurs when ADP is added.

Since the steady-state extent of reduction of cytochrome *c* expresses a ratio of velocity constants, we may compare it with the ratio of the transient rate of oxidation of *c* to the steady-state electron flux in the respiratory chain. Here we find a paradox (Chance, 1965): the ratio of transient to steady-state rates is $380:5$, or 76 , in state 3 and $380:0.5$, or 760 , in state 4, but the ratio of oxidized to reduced cytochrome *c* in the steady state is much smaller, about $5:1$ for rat-liver mitochondria or $3:1$ for flight-muscle sarcosomes in state 4. It is apparent that a control of cytochrome *c* oxidation is operative in the steady state that is not observed in the transient response of the chain. In fact, the steady-state and transient conditions may differ energetically and chemically as well. At the time of addition of oxygen to the anaerobic mitochondria in state 5, the system is at a low energy level. After a steady state of electron flow is reached, a higher energy level may obtain and energy-coupling mechanisms may then control the rate of cytochrome oxidation.

Control mechanisms for electron transport. The

Table 2. Steady states of cytochrome *c* or *f* in active (state 3) and resting (state 4) conditions due to ADP addition

Material	Source	Substrate	Inhibitor	Percentage reduction			Reference
				State 3	State 4	Difference	
Mitochondria	Rat liver	Succinate	None	13	15	+2	Chance & Williams (1955c)
Sarcosomes	Rat heart	α -Oxoglutarate	None	15	8	-7	Chance & Baltscheffsky (1958)
Sarcosomes	Locust flight muscle	Glycerol 1-phosphate	None	20	28	+8	Klingenberg & Schollmeyer (1961)
Mitochondria	Rat liver	Succinate + glutamate	Azide (300 μ M)	80	35	-45	Wilson & Chance (1966)
Chloroplasts	Spinach leaf	Diquat	None	36	21	-15	Avron (1966)

chemical mechanism for control of electron transport that is implied by eqn. (8) may be considered in more detail in eqns. (10), (11) and (12). Reduced cytochrome *c* in the uninhibited form $c'' \cdot I$ may be rapidly oxidized in its initial reaction with cytochrome *a*, as, for example, on addition of oxygen to the reduced carriers of anaerobic mitochondria (see eqns. 4, 5 and 6). This is a fast reaction (half-time of 1.8 msec.) in which energy may be conserved only in a special form, for example, in a conformation change of cytochrome itself as denoted by *:

The intermediate $(c'' \cdot I)^*$ is more slowly reduced by cytochrome c_1 (the half-times are about 0.1 sec. in submitochondrial particles; reduction times of cytochromes in mitochondria are difficult to measure because of substrate permeability), and energy conversion in a chemical (covalent) bond is indicated by $\sim I$:

$c'' \sim I$ is unreactive towards a''' and thus the reaction of eqn. (10) cannot proceed further. Therefore recovery of $c'' \cdot I$ from $c'' \sim I$ by such energy-requiring mitochondrial functions as phosphorylation of ADP, cation accumulation or reversed electron transport must occur before the reaction can continue. The rate of conversion of $c'' \sim I$ into $c'' \cdot I$ (k_6) controls electron flow at one site in the respiratory chain:

Similar reactions supplement this control at other sites.

An alternative mechanism appears to involve a membrane pH gradient as a factor in the control of electron transport (Mitchell & Moyle, 1965). However, recent experiments indicate that the membrane pH gradient is less than 0.01 unit in the presence of a permeant anion such as inorganic phosphate (Chance & Mela, 1966), and in view of the relatively low sensitivity of electron transport to pH variation (Chance & Conrad, 1959) it is not evident how electron transport can be controlled according to the proton-gradient theory.

We may now consider a number of physical and chemical mechanisms for the control of electron transfer between cytochromes *c* and *a* (Scheme 5) among which a purely chemical reaction is illustrated by the simple formulation $c \sim I$ (eqns. 11 and 12). One chemical mechanism is that eqn. (11) represents the rupture of one of the haem linkages of cytochrome *c* with substitution of a ligand of lower reactivity, possibly resulting in an altered absorp-

Scheme 5. Possible mechanisms for control of electron transfer between cytochromes *c* and *a*, involving thermal agitation leading to rotation or translation of the molecules with respect to one another, variations of ligand binding and haem linkage to the iron atom of the cytochromes, conformation change in the helical structure of the protein, and variation of tunnelling barrier width owing to membrane distortion and other factors.

tion spectrum for cytochrome *c*. This can be tested experimentally on the assumption that the addition of ADP and inorganic phosphate, or of an uncoupler of oxidation from phosphorylation, would destroy the linkage to the haem that was responsible for the control of electron transport. A further assumption is that ATP activation of reversed electron transport also forms a 'high-energy' compound involving such a linkage. Thus the spectrum representing the difference between the energized and de-energized (high- and low-energy) states of cytochrome *c* could indicate an exchange of ligand. Though rather striking results have been obtained with cytochrome *b*, the spectrum of cytochrome *c* shows the clear α_1 and α_2 peaks and other characteristics to be identical in the two energy states (Chance & Schoener, 1966). Thus, if a ligand shift does occur, it does not show in the spectrum.

The second possibility is that the inactive *c* ~ I represents a conformation change, and that the conformation of cytochrome *c* differs in the resting and active states. The relatively slow process of electron transport in the respiratory chain affords ample time for conformation changes of the type suggested for soluble cytochrome *c* in the oxidized-reduced transition, although such changes may be difficult to detect in mitochondria. Though changes in reactivity towards carboxymethylation were observed *in vitro* by Matsubara *et al.* (1965), their methods are not sufficiently specific to allow of unambiguous application to reactions with cytochromes within the structure of the mitochondrion. Optical methods are not sensitive enough to detect such changes in the highly light-scattering suspensions of mitochondria, nor are magneto-optical

techniques sufficiently refined to detect rotatory dispersions of the type attributable to conformation changes in the high-to-low energy transition. We are therefore without a convenient means of proving or disproving whether or not a protein conformation change is involved in the control of electron-transport.

The third possibility is that a variable barrier may exist between cytochromes *c* and *a*. Such a barrier was proposed as a part of the tunnelling process in the low-temperature electron transfer between cytochrome *c*₄₂₃₋₅ and bacterial chlorophyll. In that case, the barrier probably is not involved in the energy-conservation aspects of electron transport, but instead acts as a purely structural hindrance. However, we may consider whether a tunnelling barrier could constitute a rate-determining step in electron transfer between cytochromes, for example between *c* and *a*. It is apparent from the temperature-sensitivity of the intercytochrome reaction that a 'pure' tunnelling process is not involved; otherwise, a temperature-insensitivity would be observed. However, a form of tunnelling reaction considered in detail by DeVault & Chance (1966b) treats the case where the effective barrier width can be altered by thermal agitation. In preliminary calculations, values for the barrier height seemed unreasonably large for the cytochrome *c* reaction in bacteria; nevertheless, this mechanism for the control of electron transport remains for further consideration and more detailed calculations.

A fourth possibility is that of structural control of activity, as demonstrated for tetrameric forms of haemoglobin in the elegant work of Perutz (1965) and his colleagues, and for the monomeric form by recent studies of the reaction of crystalline ferrimyoglobin and azide (Chance, Ravilly & Rumen, 1966). In the latter case, we have found a greatly diminished reaction rate in the crystalline material as compared with that in solution. It appears that the reactivity is controlled by the rigidity of the protein structure, which determines the probability that a conformation of the protein appropriate to ligand binding occurs. This conformation, however, need only be achieved in the transition state ('transitional conformation'; see Chance & Watson, 1966); the initial and final forms of the protein in this sort of reaction are isomorphous.

Such studies have done much to clarify the factors involved in the binding of small ligands. The applicability of these results to cytochrome *c* electron transport depends on whether electron conduction through the haem-linked group to the periphery of the protein is subject to the same controls as is the binding of small ligands.

The fifth possibility involves gross structural changes in the surrounding mitochondria. Two lines of evidence call our attention to such structural

Fig. 7. Comparison of specific spectral changes due to an electron-transfer component (cytochrome *c*) (top trace), non-specific light-absorption changes (middle trace) and rate of respiration (bottom trace) in a suspension of rat-liver mitochondria activated by ADP, Ca^{2+} and dicoumarol. The top trace is recorded with a double-beam spectrophotometer set at $550\text{ m}\mu$ – $540\text{ m}\mu$; the sensitivity scale is indicated on the right-hand side. The middle trace was recorded at $540\text{ m}\mu$ and at a lower sensitivity, as indicated on the right; the lower trace is the derivative of the change in the oxygen concentration and the value of the rate is indicated by the numbers on the chart. The rat-liver mitochondria (approx. 4 mg. of protein/ml.) are suspended in a 0.225 M-mannitol–0.075 M-sucrose in 10 mM-tris–HCl buffer, pH 7.4; in addition Mg^{2+} (5 mM), phosphate (3 mM) and succinate (3 mM) are present. The concentrations of ADP, Ca^{2+} and dicoumarol added are as indicated. The temperature is 23° . For further experimental details, see Chance (1963).

differences in the resting state 4 and the active state 3. The first is based on experiments in which light-transmission through the mitochondrial suspension is measured at a wavelength at which no specific absorption bands appear. Such a wavelength is $540\text{ m}\mu$, which is isosbestic for both the state 4–state 3 transition and the oxygen–nitrogen transition. If non-specific light-transmission is recorded at this wavelength, characteristic spectral changes are observed in the state 4–state 3 transition (Chance & Packer, 1958; Lehninger, 1962). We may take as an example the experiment shown in Fig. 7, where a state 4–state 3 transition was caused by the addition of substances of three different types: ADP, Ca^{2+} and dicoumarol. Here the non-specific spectral changes are recorded on a scale one-fifth as sensitive as that employed for the cytochrome *c* responses. Though the increase in non-specific light-absorption is small on the addition of ADP, it is considerable when Ca^{2+} and dicoumarol are added, and appears to be closely synchronized with the spectral changes due to cytochrome *c* oxidation, and with the respiratory rate indicated by the derivative trace on the bottom line of the graph. The return of the non-specific light-absorption trace as the added ADP or Ca^{2+} is expended appears to be somewhat less abrupt than the return of the cytochrome *c* trace. However, the general

correlation between the changes in non-specific light-absorption, overall respiratory rate and oxidation state of cytochrome *c* under these conditions is impressive, and closely relates a change of mitochondrial structure with the state 4–state 3 transition.

Further evidence for structural changes is afforded by electron microscopy. Hackenbrock (1966) has demonstrated highly significant changes in the nature of both the matrix space and the cristae in the state 4–state 3 transition. If mitochondria allowed to stand in state 4 for 15 min. are then supplemented with ADP for 45 sec., striking changes in the electron micrographs are evident. The typical form of rat-liver mitochondrion, with well-defined cristae and an electron-translucent matrix space, is rapidly converted into a form showing a highly granular matrix space with large distortions in the crystal structure. We may correlate the greater density of the matrix space with a greater non-specific light-absorption of the mitochondrial suspension. Though both the light-absorption and electron-micrographic changes coincide closely with the state 4–state 3 transition, both seem to lag behind the electron carriers in the reverse (state 3–state 4) transition, in which other factors may be involved.

Control of electron transport by energy-coupling

reactions can be observed in submitochondrial particles derived from heart mitochondria by disruption with ultrasonic vibrations (Lee & Ernster, 1966). When maximal control is induced by a supplement of oligomycin, a portion of the heterogeneous biochemical population shows extremely slow electron transport upon addition of NADH to the cyanide-inhibited system; the rate is one-sixteenth that of the same material supplemented with an uncoupling agent (C. P. Lee, L. Ernster & B. Chance, unpublished observations). Thus the mechanisms proposed for the control of electron transport in mitochondria should be applicable to the control of electron transport in the vesicular structure of submitochondrial particles as well. Electron-microscopic determination of structural alterations in the coupled and uncoupled state will test the above hypothesis in a particularly crucial manner.

Two general categories of control emerge from this discussion. One is based on the observation of diminished reaction rates in crystalline ferrimyoglobin, which suggests that changes in protein structure that alter its flexibility may control its reactivity as well. The reaction of the energy-conserving $\sim I$ may be of this type. The other has its roots in Keilin & Hartree's views on the mutual accessibility of the components of the respiratory chain. If the gross structural changes in the mitochondrial membrane that coincide with the transition from the inactive state 4 to the active state 3 either increase the freedom of rotation of the cytochromes or decrease the width of the tunnellable barrier, a high degree of control of electron transport would be possible.

Further evidence for this type of control may come from the variable response of cytochrome *b* in intact mitochondria and in submitochondrial particles, particularly those of the Keilin & Hartree type. The conversion of mitochondrial cristae into vesicular bodies, which has been considered at length by Green & Perdue (1966), causes considerable inhibition in the rate of cytochrome *b* reduction (Chance, 1952). It may be presumed that this conversion gives rise to structural dislocations of the respiratory chain that result in diminished electron-transport capabilities.

Relationship of control of electron transport to energy conservation. In these discussions we have stressed the physical and chemical mechanisms for the control of electron transfer, and have emphasized that compounds formed in energy-conserving processes may play an important role in this control. Eqns. (11) and (12), however, indicate that energy conservation precedes the formation of the rate-controlling intermediate. In submitochondrial particles the rate of eqn. (12) is as high in the absence of ADP and phosphate as when these

substances are present. Of the various control mechanisms described above, all except that based on the integrity of the structure of the mitochondrion could operate effectively in submitochondrial particles as well.

Summary

This Lecture has attempted to evaluate the present status of experimental work on, and development of ideas about, the nature and control of electron-transport mechanisms in multi-enzyme sequences of cytochromes. We have been able to identify four levels of electron-transfer activity involving cytochromes of types *c* or *f*. The first two processes are observed in photosynthetic bacteria, the latter pair in mitochondria.

The first process is a temperature-insensitive reaction observed to occur in *Chromatium* with a half-time of 2 msec. at 34°K, which would probably occur almost as rapidly at 0°K. One mechanism suggested for this reaction involves electron conduction from the haem of cytochrome *c* via its linkages to the periphery of the protein and eventually to the electron acceptor, chlorophyll, by means of a quantum-mechanical tunnelling of electrons through the barrier between the two. This reaction is, most probably, an electron transfer involving no thermal agitation, bond-breaking or conformation change.

A second type of electron transport is observed in photosynthetic systems at room temperature and is much more rapid, with half-times of about 2 μ sec. for cytochrome *c* in *Chromatium* or 14 μ sec. for cytochrome *f* in *Porphyridium*. These are the fastest biological reactions yet directly recorded. They require thermal agitation and may or may not be accompanied by electron tunnelling. It is possible that this type of electron-transport reaction is too rapid to permit energy conservation, and serves only to transfer the electron-accepting site from chlorophyll to a more convenient location for thermal reactions in the photosynthetic structure.

The third, much slower, reaction is observed in electron transport between cytochromes of the respiratory chain in mitochondria. Here thermal agitations are necessary, not only to deliver the electron to the acceptor, but also to allow the acceptor to receive it from the donor. This reaction has a half-time of 1.8 msec. at 33°.

The fourth reaction is observed in the steady state of electron transport in the respiratory chain, and proceeds at rates as low as 0.5 sec.⁻¹ in the controlled state 4. Although the cytochrome turnover numbers are very small compared with their oxidation rates, the cytochromes are appreciably reduced in the resting state of electron flow (state 4)

and may become even more reduced in the active state (state 3). Thus our attention is called to special factors controlling electron transport in reactions involved in energy conservation.

Five mechanisms for the control of electron transport in energy-conserving systems are considered: first, by chemical modification of the cytochrome, for example by ligand substitution; secondly, by a protein conformation change that decreases the activity of the cytochrome; thirdly, by an increase in the width or height of an electron-tunnelling barrier; fourthly, by an increase in the rigidity of the protein structure that might inhibit electron flow in a manner similar to that by which it inhibits the binding of small ligands in ferrimyoglobin; fifthly, by a generalized structural rearrangement of the components of the mitochondrial electron-transport chain.

Spectroscopic data do not as yet afford support for the first mechanism, involving ligand binding to the haem. The available methods are not adequate to determine whether reaction mechanisms of the second type, observed in cytochrome *c* in solution, are operative within the mitochondrial structure. Evidence for a tunnelling barrier in bacterial electron transport calls our attention to the third possibility. Reaction mechanisms of the fourth type are found to control the reactivity of small ligands towards ferrimyoglobin in the crystalline state, and control of haemoprotein reactivity by transitional alterations in the flexibility of its structure appears feasible. Experimental evidence is available for the fifth process, involving structural changes in the mitochondrial framework, but the kinetic correlation of these changes with the electron-flow rate is incomplete.

Though no one of these mechanisms seems, in itself, adequate to explain the experimental evidence for control of electron flow, two combinations of these possibilities are of great interest: a ligand interaction that alters protein flexibility, or a gross structural change of the mitochondrial membrane that alters protein mobility or tunnelling barrier width.

I had the good fortune to be a research student in the 1930s under F. J. W. Roughton and G. A. Millikan. The curriculum afforded me a unique opportunity to roam freely in the natural sciences and to stress physics and mathematics. Though my predoctoral contacts seldom reached as high as Professor Keilin, Professor Dixon or Dr Hill, my postdoctoral contacts did. In 1947–48 after a most fruitful interval with Professor Theorell, I spent several months at the Molteno Institute, and it was my privilege to be visited during Professor Keilin's post-tea time peregrinations around the laboratory. All his words were to be remembered; all were based

on his own immense experimental experience, his deep insight and his intuition for important biochemical problems. In the years that followed, no visit to Cambridge was complete without a few hours of intensive discussion with Professor Keilin. He was solicitous, not only of one's scientific progress but of one's personal problems as well. It is a tribute to his genius that the Molteno Institute rose and stood out like a bright star among the many brilliant lights of Cambridge. It was my great pleasure to have been a small part of this, and my great honour to present the second Keilin Memorial Lecture.

This Lecture represents a summary of 'work in progress' to which many have contributed, particularly Don DeVault, William Hildreth, William Parson and Mitsuo Nishimura on cytochrome oxidation in photosynthetic micro-organisms, Brigitte Schoener on cytochrome oxidation in mitochondria, and Takashi Yonetani on cytochrome *c*-peroxidase kinetics. Victor Legallais has been responsible for the design and development of much of the instrumentation that made these studies possible.

The manuscript has been greatly improved by thoughtful suggestions from Joan Keilin, E. C. Slater and E. F. Hartree, to whom my most sincere thanks are due.

REFERENCES

- Altschul, A. M., Abrams, R. & Hogness, T. R. (1940). *J. biol. Chem.* **136**, 777.
- Avron, M. (1966). *Brookhaven Symp. Biol.* **19**, 149.
- Avron, M. & Chance, B. (1966). In *Currents in Photosynthesis*, p. 455. Ed. by Thoma, J. B. & Godheer, J. C. Rotterdam: A. B. Donker.
- Ball, E. G. (1938). *Biochem. Z.* **295**, 262.
- Baltscheffsky, H. & Arwidsson, B. (1962). *Biochim. biophys. Acta*, **65**, 425.
- Bartsch, R. (1966). *Proc. 66th annu. Meet. Amer. Soc. Microbiol., Los Angeles*.
- Beetlestone, J. (1960). *Arch. Biochem. Biophys.* **89**, 35.
- Chance, B. (1943). *J. biol. Chem.* **151**, 553.
- Chance, B. (1951a). *Advanc. Enzymol.* **10**, 153.
- Chance, B. (1951b). In *Enzymes and Enzyme Systems*, p. 95. Ed. by Edsall, J. Cambridge, Mass.: Harvard University Press.
- Chance, B. (1952). *Nature, Lond.*, **169**, 215.
- Chance, B. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 300. Ed. by Liébecq, C. Liège: Vaillant-Carmanne.
- Chance, B. (1963). In *Energy-Linked Functions of Mitochondria*, p. 253. Ed. by Chance, B. New York: Academic Press Inc.
- Chance, B. (1964). In *Rapid Mixing and Sampling Techniques in Biochemistry*, p. 125. Ed. by Chance, B., Gibson, Q. H., Eisenhardt, R. & Lonberg-Holm, K. K. New York: Academic Press Inc.
- Chance, B. (1965). In *Oxidases and Related Redox Systems*, vol. 1, p. 67. Ed. by King, T. E., Mason, H. S. & Morrison, M. New York: John Wiley and Sons Inc.
- Chance, B. & Baltscheffsky, M. (1958). *Biochem. J.* **68**, 283.
- Chance, B. & Conrad, H. (1959). *J. biol. Chem.* **234**, 1568.
- Chance, B. & DeVault, D. (1964). *Dtsch. Bunsenges. physik. Chem.* **68**, 722.

- Chance, B., DeVault, D., Hildreth, W. W., Parson, W. & Nishimura, M. (1966a). *Brookhaven Symp. Biol.* **19**, 115.
- Chance, B., Estabrook, R. W. & Yonetani, T. (Eds.) (1966b). *Hemes and Hemoproteins*. New York: Academic Press Inc.
- Chance, B. & Mela, L. (1966). *J. biol. Chem.* **241**, 4588.
- Chance, B. & Nishimura, M. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 19.
- Chance, B. & Packer, L. (1958). *Biochem. J.* **68**, 295.
- Chance, B., Ravilly, A. & Rumen, N. (1966). *J. molec. Biol.* **17**, 525.
- Chance, B. & Schindler, F. J. (1965). In *Oxidases and Related Redox Systems*, vol. 2, p. 921. Ed. by King, T. E., Mason, H. S. & Morrison, M. New York: John Wiley and Sons Inc.
- Chance, B., Schleyer, H. & Legallais, V. L. (1963). In *Microalgae and Photosynthetic Bacteria* (Special Issue of *Plant & Cell Physiol.*), p. 337.
- Chance, B. & Schoener, B. (1966). *J. biol. Chem.* **241**, 4567.
- Chance, B., Schoener, B. & DeVault, D. (1965). In *Oxidases and Related Redox Systems*, vol. 2, p. 907. Ed. by King, T. E., Mason, H. S. & Morrison, M. New York: John Wiley and Sons Inc.
- Chance, B. & Smith, L. (1955). *Nature*, **175**, 803.
- Chance, B. & Watson, H. C. (1966). In *Hemes and Hemoproteins*, p. 149. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Chance, B. & Williams, G. R. (1955a). *J. biol. Chem.* **217**, 383.
- Chance, B. & Williams, G. R. (1955b). *J. biol. Chem.* **217**, 395.
- Chance, B. & Williams, G. R. (1955c). *J. biol. Chem.* **217**, 409.
- Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.
- Clayton, R. K. (1962). *Photochem. Photobiol.* **1**, 313.
- DeVault, D. (1964). In *Rapid Mixing and Sampling Techniques in Biochemistry*, p. 165. Ed. by Chance, B., Gibson, Q. H., Eisenhardt, R. & Lonberg-Holm, K. K. New York: Academic Press Inc.
- DeVault, D. & Chance, B. (1966a). *Abstr. 10th Annu. Meet. biophys. Soc., (U.S.)*, no. WC-4, p. 20.
- DeVault, D. & Chance, B. (1966b). *Biophys. J.* **6**, 825.
- Dickerson, R. E., Kopka, M. L., Weinzierl, J., Varnum, J. & Borders, C. L. (1966). In *Hemes and Hemoproteins*, p. 365. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Dixon, M., Hill, R. & Keilin, D. (1931). *Proc. Roy. Soc. B*, **107**, 286.
- Duysens, L. N. M. (1954). *Nature, Lond.*, **173**, 692.
- Duysens, L. N. M., Ames, J. & Kamp, B. M. (1961). *Nature, Lond.*, **190**, 510.
- Duysens, L. N. M. (1966). *Brookhaven Symp. Biol.* **19**, 71.
- Estabrook, R. W. (1958). *J. biol. Chem.* **230**, 735.
- Evans, H. J. & Gergeley, J. (1949). *Biochim. biophys. Acta*, **3**, 188.
- George, P. (1956). In *Currents in Biochemical Research*, p. 338. Ed. by Green, D. E. New York: Interscience Publishers Inc.
- Gibson, Q. H. & Greenwood, C. (1963). *Biochem. J.* **86**, 541.
- Green, D. E. & Perdue, J. F. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 1295.
- Hackenbrock, C. R. (1966). *J. Cell Biol.* **30**, 269.
- Harbury, H. A., Myer, Y. P., Murphy, A. J. & Vinogradov, S. N. (1966). In *Hemes and Hemoproteins*, p. 415. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Hettinger, T. P., Langer, M. W., Vinogradov, S. N. & Harbury, H. A. (1966). *Fed. Proc.* **25**, 648.
- Hildreth, W. W., Avron, M. & Chance, B. (1966). *Abstr. 10th Annu. Meet. biophys. Soc., (U.S.)*, no. WC-3, p. 20.
- Hill, R. & Bhagvat, K. (1939). *Nature, Lond.*, **143**, 726.
- Hill, R. & Keilin, D. (1930). *Proc. Roy. Soc. B*, **107**, 286.
- Hill, R. & Keilin, D. (1933). *Proc. Roy. Soc. B*, **114**, 104.
- Keilin, D. (1925a). *Nature, Lond.*, **115**, 446.
- Keilin, D. (1925b). *Proc. Roy. Soc. B*, **98**, 312.
- Keilin, D. (1929). *Proc. Roy. Soc. B*, **104**, 206.
- Keilin, D. (1930). *Proc. Roy. Soc. B*, **106**, 418.
- Keilin, D. (1966). *The History of Cell Respiration and Cytochrome*. Cambridge: Cambridge University Press.
- Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc. B*, **122**, 298.
- Keilin, D. & Hartree, E. F. (1939). *Proc. Roy. Soc. B*, **127**, 167.
- Keilin, D. & Hartree, E. F. (1940). *Proc. Roy. Soc. B*, **129**, 277.
- Keilin, D. & Hartree, E. F. (1949). *Biochem. J.* **44**, 205.
- Keilin, D. & Mann, T. (1937). *Proc. Roy. Soc. B*, **122**, 119.
- King, T. E., Kuboyama, M. & Takemori, S. (1965). In *Oxidases and Related Redox Systems*, vol. 2, p. 707. Ed. by King, T. E., Mason, H. S. & Morrison, M. New York: John Wiley and Sons Inc.
- Klingenberg, M. & Schollmeyer, P. (1961). *Proc. 5th int. Congr. Biochem., Moscow*, vol. 5, p. 46. Ed. by Slater, E. C. London: Pergamon Press Ltd.
- Kowalsky, A. (1966). In *Hemes and Hemoproteins*, p. 529. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Kowalsky, A. & Cohn, M. (1964). *Annu. Rev. Biochem.*, **33**, 481.
- Lardy, H. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 287. Ed. by Liébecq, C. Liège: Vaillant-Carmanne.
- Lee, C. P. & Ernster, L. (1966). In *Symp. on Regulation of Metabolic Processes in Mitochondria*, p. 218. Ed. by Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co. (Biochim. biophys. Acta Library, vol. 7).
- Lehninger, A. L. (1962). *Physiol. Rev.* **42**, 467.
- Ludwig, G. D. & Kuby, S. A. (1955). *Fed. Proc.* **14**, Abstr. 800.
- Lundegårdh, H. (1961). *Nature, Lond.*, **192**, 243.
- Maley, G. F. & Lardy, H. A. (1954). *J. biol. Chem.* **210**, 903.
- Margoliash, E. (1966). In *Hemes and Hemoproteins*, p. 371. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Margoliash, E. & Schejter, A. (1966). In *Advances in Protein Chemistry*, vol. 21, p. 114. Ed. by Anfinsen, C. B., Anson, M. L., Edsall, J. T. & Richards, F. M. New York: Academic Press Inc.
- Marsh, P. B. & Goddard, D. R. (1939). *Amer. J. Bot.* **26**, 724.
- Matsubara, H., Ando, K. & Okunuki, K. (1965). *Proc. imp. Acad., Japan*, **41**, 408.
- Mitchell, P. & Moyle, J. (1965). *Nature, Lond.*, **208**, 147.
- Morita, S., Edwards, M. & Gibson, A. J. (1965). *Biochim. biophys. Acta*, **109**, 45.
- Nicholls, P. (1964). *Arch. Biochem. Biophys.*, **106**, 25.
- Nielsen, S. O. & Lehninger, A. L. (1954). *J. Amer. chem. Soc.* **76**, 3860.

- Olson, J. M. & Chance, B. (1960). *Arch. Biochem. Biophys.* **88**, 26.
- Parson, W. (1966). *Brookhaven Symp. in Biol.* **19**, 195.
- Perutz, M. F. (1965). *J. molec. Biol.* **13**, 646.
- Roughton, F. J. W. & Chance, B. (1961). In *Techniques of Organic Chemistry*, vol. 8, part 2, p. 703. Ed. by Freiss, S. L., Lewis, E. S. & Weissberger, A. New York: Interscience Publishers Inc.
- Shashoua, V. L. & Estabrook, R. W. (1966). In *Hemes and Hemoproteins*, p. 427. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Shibata, K. (1935). *Ergebn. Enzymforsch.* **4**, 348.
- Shibata, K. & Tamiya, H. (1930). *Acta Phytochim., Tokyo*, **5**, 23.
- Slater, E. C. (1950a). *Biochem. J.* **46**, 484.
- Slater, E. C. (1950b). *Nature, Lond.*, **166**, 982.
- Slater, E. C. (1953). *Nature, Lond.*, **172**, 975.
- Slater, E. C. (1955a). *Biochem. J.* **59**, 392.
- Slater, E. C. (1955b). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 264. Ed. by Liébeq, C. Liège: Vaillant-Carmanne.
- Smith, L. (1954). *Bact. Rev.* **18**, 106.
- Smith, L. & Conrad, H. (1961). In *Haematin Enzymes*, part 1, p. 276. Ed. by Falk, J. E., Lemberg, M. R. & Morton, R. K. Oxford: Pergamon Press Ltd.
- Stotz, E., Altschul, A. M. & Hogness, T. R. (1938). *J. biol. Chem.* **124**, 745.
- Theorell, H. (1935). *Biochem. Z.* **279**, 463.
- Theorell, H. (1936). *Biochem. Z.* **285**, 207.
- Theorell, H. (1938). *Biochem. Z.* **298**, 247.
- Theorell, H. (1939). *Enzymologia*, **6**, 88.
- Theorell, H. (1941). *Enzymologia*, **10**, 250.
- Theorell, H. (1944). *Mitt. Naturf. Ges. Bern*, p. 85.
- Theorell, H. (1956). *Science*, **124**, 467.
- Theorell, H. & Åkesson, Å. (1941). *J. Amer. chem. Soc.* **63**, 1804.
- Townes, C. H. & Schawlow, A. L. (1958). *Phys. Rev.* **112**, 1940.
- Tsou, C. L. (1952). *Biochem. J.* **50**, 493.
- Urry, D. W. & Eyring, H. (1965a). *J. theoret. Biol.* **8**, 198.
- Urry, D. W. & Eyring, H. (1965b). *J. theoret. Biol.* **8**, 214.
- Vredenberg, W. J. & Duysens, L. N. M. (1964). *Biochim. biophys. Acta*, **79**, 456.
- Warburg, O. (1924). *Biochem. Z.* **152**, 479.
- Warburg, O., Negelein, E. & Haas, E. (1933). *Biochem. Z.* **266**, 1.
- Weiland, H. (1922). *Ergebn. Physiol.* **20**, 477.
- Wilson, D. F. & Chance, B. (1966). *Biochem. biophys. Res. Commun.* **23**, 751.
- Witt, H. T., Müller, A. & Rumberg, B. (1963). *Nature, Lond.* **197**, 987.
- Witt, H. T., Rumberg, B., Schmidt-Wende, F., Siggel, U., Skena, B., Vater, G. & Weinbasch, J. (1965). *Angew. Chem.* **77**, 821.
- Yakushiji, E. & Okunuki, K. (1940). *Proc. imp. Acad., Japan*, **16**, 299.
- Yonetani, T. & Ray, G. S. (1966). *J. biol. Chem.* **241**, 700.
- Yonetani, T., Schleyer, H., Chance, B. & Ehrenberg, A. (1966). In *Hemes and Hemoproteins*, p. 293. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Zweig, G., Shavit, N. & Avron, M. (1965). *Biochim. biophys. Acta*, **109**, 332.