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The Morphological Site of Synthesis of Cytochrome *c* in Mammalian Cells (Krebs Cells)

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In Krebs ascites-tumour cells, cytochrome *c* is segregated in the mitochondria and the level in microsomes could not be measured. At 22° in glucose–buffer Krebs cells synthesized a spectrum of proteins including cytochrome *c*. Mild osmotic shock in the presence of ribonuclease had little effect on incorporation of [^{14}C]-leucine or [^{14}C]valine into mixed mitochondrial protein but strongly inhibited synthesis of non-mitochondrial cytoplasmic proteins. Under these conditions, labelling of cytochrome *c* was also strongly inhibited. After pulse labelling of Krebs cells at 22° for 10min. the cytcchrome radioactivity found in mitochondria was higher than in microsomes. After addition of unlabelled amino acid as 'chase' there was 137% increase in radioactivity of cytochrome *c* but only a 3% increase in radioactivity of whole-cell protein. It is concluded that the peptide chain of cytochome *c* is synthesized on cytoplasmic ribosomes. Mitochondria therefore do not have the character of self-replicating entities, but are formed by the co-operative function of messenger RNA of cytoplasmic ribosomes and, possibly, of intramitochondrial messenger derived from the mitochondrial DNA.

Cytochrome *c* is a typical mitochondrial protein; it is an essential component of the electron-transport chain and it does not occur in significant quantity elsewhere in the cell (Chance & Hess, 1959; Freeman, 1965). It was therefore with some surprise that we found cytochrome *c* to be unlabelled after incubation of isolated mitochondria with radioactive amino acids (Roodyn, Suttie & Work, 1962). Under the conditions of these experiments there was linear increase in the radioactivity of mitochondrial protein with time of incubation for at least 1hr. The labelling was shown to be within protein chains rather than terminal (Suttie, 1962) and was dependent on the maintenance of oxidative phosphorylation. There have been suggestions (Sandell, Löw & Decken, 1966) that the labelling that occurs under the conditions of incubation *in vitro* is due to bacterial contamination, but Roodyn (1965, 1966) has shown that this is a most unlikely explanation. The paradoxical situation therefore exists that a protein found only in mitochondria is not labelled *in vitro* under conditions where mitochondrial proteins are being synthesized.

Some of the arguments for the existence of two separate sites for the synthesis of mitochondrial proteins have been presented recently (Haldar, Freeman & Work, 1966, 1967) and we now present evidence to show that the peptide chain of cytochrome *c* is synthesized on extramitochondrial cytoplasmic ribosomes. Evidence has already been presented that the insoluble proteins of the mitochondria are synthesized within these organelles (Haldar *et al.* 1967), and the present results force us to the conclusion that mitochondrial biogenesis involves integrated protein synthesis governed by two independent but interdependent genetic centres (cf. Haldar *et al.* 1966).

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MATERIALS AND METHODS

Krebs cells. The Krebs II tumour was maintained in mice as described by Martin, Malec, Sved & Work (1961). Cells were collected, washed and counted as described by these authors.

Buffers. The three buffers used were those described by Martin *et al.* (1961): phosphate-buffered saline, Ca^{2+}- and Mg^{2+}-free phosphate saline and Earle's medium.

Radioactive isotopes. DL-[1-^{14}C]Valine and DL-[1-^{14}C]-leucine were obtained from The Radiochemical Centre, Amersham, Bucks. The valine had a specific activity of 5mc/m-mole and the leucine a specific activity of 12 mc/m-mole.

Isolation and purification of mouse cytochrome c. A weighed amount of mouse liver was homogenized in one-quarter its own weight of water and 1·2vol. of 0·5N-H_2SO_4 was added. The pH was adjusted to 3·0 by addition of aq. 2N-NH_3 and after 15min. the precipitate was removed (6000*g* for 10min.). The supernatant was neutralized with N-NaOH and cooled to 4°. Ammonium sulphate was added (0·55g./ml.) and the solution left overnight at 4°. Precipitated protein was removed (6000*g* for 10min.) and the

supernatant was filtered through Whatman no. 3 paper to remove fat. Trichloroacetic acid was added to a final concentration of 0·5% (w/v) and after 2hr. at 0° the precipitated cytochrome *c* was collected. The cytochrome was dissolved in water and dialysed against 0·05N-NH_3 at 4°, the pH was adjusted to 6·8 with 0·5N-cacodylic acid and the final purification was carried out on CM-cellulose.

Whatman CM30 CM-cellulose was suspended in 0·01M-tris–HCl buffer, pH9, and poured on to a suitable column. Crude cytochrome *c* was added and the protein eluted with the following buffers, all at 0·01M, in succession; tris, pH9·0; glycine–NaOH, pH10·3; glycine buffer, pH10·6; glycine buffer, pH10·6, containing 0·03M-NaCl; glycine buffer, pH10·6, containing 0·1M-NaCl. Cytochrome *c* was eluted with the last buffer. A red band was usually left at the top of the column, presumably polymerized cytochrome *c* (Margoliash & Lustgarten, 1962).

Radioactive cytochrome c. In experiments with Krebs cells where radioactive cytochrome *c* had to be isolated and counted, the method was as follows. Radioactive cells were spun down, then either resuspended in water + carrier cytochrome *c* or, where individual subcellular components had to be separated, mixed with unlabelled carrier cells so as to have sufficient material in each subfraction. Individual methods are indicated in the Tables. To purify the cytochrome *c*, H_2SO_4 and NH_3 were added as described for the purification procedure above and the cytochrome was taken through all stages to the trichloroacetic acid precipitation. The precipitate was taken up in water and dialysed against a large excess of 0·05N-NH_3. For this dialysis specially prepared acetylated Visking tubing was used. Cytochrome *c* has a sufficiently low molecular weight to pass slowly through ordinary dialysis tubing, but Craig (1960) has shown that the pore size can be reduced by acetylation, and by following Craig's method tubing was prepared that was impermeable to cytochrome *c* but still readily permeable to valine or leucine. The radioactive cytochrome *c* was finally purified on CM-cellulose as described above and the cytochrome band was usually collected in two lots, which were counted separately. For counting, the amount of cytochrome *c* was estimated spectrophotometrically (Paul, 1955; Van Gelder & Slater, 1962) and Triton X-100 then was added to a final concentration of 0·1%. A 2·5ml. sample was mixed with 1g. of fluorescence-grade anthracene and counted in a Packard Tri-Carb liquid-scintillation counter (Steinberg, 1960). Correction for quenching was made by re-counting after adding standard in the form of ^{14}C-labelled amino acid. Alternatively, the cytochrome *c* was precipitated without detergent by the addition of trichloroacetic acid to 5% (w/v), collected on a Millipore filter and counted on a Nuclear–Chicago low-background gas-flow counter. Method 1 had an efficiency of about 23% and method 2 an efficiency of about 18%. The low efficiency of method 1 is due to quenching by cytochrome *c*.

RESULTS

Purification and characterization of mouse cytochrome c. Full details of the methods of purification of cytochrome *c* from Krebs II mouse ascites-tumour cells and from mouse liver are given in the Materials and Methods section. The initial extraction from either source followed the methods of Keilin & Hartree (1937) and Rosenthal & Drabkin (1943). Tissue or cells were extracted with acid ammonium sulphate at about pH2·5 and cytochrome was precipitated with trichloroacetic acid after removal of less-soluble proteins with ammonium sulphate (50g./100ml.). Final purification was by chromatography on CM-cellulose (Paléus & Theorell, 1957; Margoliash & Lustgarten, 1962). Purity was checked by starch-gel electrophoresis (Smithies, 1955) and only a single band, that of cytochrome *c*, was seen.

The amount of cytochrome *c* was calculated from the spectrum by the method of Paul (1955) corrected to the extinction coefficient determined by Van Gelder & Slater (1962). The spectrum of mouse cytochrome *c* was indistinguishable from that of horse-heart cytochrome *c* (Margoliash & Frohwirt, 1959) both in the visible and ultraviolet. The E_{550} (red.)/E_{280} (ox.) ratio was 1·21. No *N*-terminal amino acid could be detected in mouse cytochrome *c* by reaction with fluorodinitrobenzene. In this respect it resembles horse-heart cytochrome *c*, in which the *N*-terminal group is blocked by an acetyl group. The amino acid composition of mouse cytochrome *c* was determined on an auto-analyser and is given in Table 1. The values quoted are the mean of three analyses on the same hydrolysate. It will be noted that the cysteine value is low (this is usual in hydrolysates of cytochrome *c*). It is also notable that, whereas horse cytochrome *c* contains 10 threonine residues/mol. and no serine, in mouse

Table 1. *Amino acid composition of mouse cytochrome* c (*liver*) *compared with that of horse-heart cytochrome* c

Results are expressed as number of moles of each amino acid present in 100 moles of amino acid residues. The values for horse cytochrome *c* are calculated from the known sequence (Margoliash, Smith, Kreil & Tuppy, 1961).

	Mouse (found)	Horse (calc.)
Cys	0·80	1·96
Asp	9·0	7·85
Thr	6·9	9·80
Ser	1·6	0·00
Glu	9·6	10·80
Pro	2·9	3·92
Gly	14·0	11·75
Ala	8·0	5·90
Val	2·8	2·94
Met	1·8	1·96
Ile	5·0	5·90
Leu	6·4	5·90
Tyr	3·9	3·92
Phe	3·7	3·92
Lys	18·0	18·65
His	3·1	2·94
Arg	2·0	1·96

cytochrome *c* serine substitutes for 2 of the threonine residues/mol.

Localization and amount of cytochrome c *in Krebs II tumour cells.* The yield of cytochrome *c* from 1×10^{10} Krebs cells was about 1·6mg. This may be compared with the value of 1·2mg. calculated by Chance & Hess (1959) for Lettré hyperdiploid Ehrlich ascites-tumour cells. As already shown by Freeman (1965), most of this cytochrome *c* is found in the mitochondria provided that cells are disrupted in iso-osmotic sucrose. The level of cytochrome *c* in the microsomal fraction was below the level of detection.

Labelling of cytochrome c *with* [^{14}C] *leucineor* [^{14}C]*valine at* 22°. As already explained (Haldar *et al.* 1967) protein synthesis by Krebs-cell suspensions proceeds satisfactorily at 22° and this is the preferred temperature for kinetic experiments. It was necessary, however, to check that cytochrome *c* was one of the proteins synthesized under these conditions. Table 2 shows that at 22° the rate of incorporation of [^{14}C]leucine into cytochrome *c* in a suspension of Krebs cells was much the same as the rate of incorporation into whole protein. In a second similar experiment [^{14}C]valine was used instead of [^{14}C]leucine. The higher specific activity of [^{14}C]leucine-labelled cytochrome *c* is in accordance with expectation having regard to the greater amount of leucine in mouse cytochrome *c* (Table 1).

Effect of ribonuclease and osmotic shock on labelling of cytochrome c. It has been established by Haldar *et al.* (1967) that Krebs cells can be made permeable to ribonuclease by mild osmotic shock. The ribonuclease selectively inhibits synthesis of microsomal protein and has relatively less effect on the synthesis of mitochondrial protein. It was therefore decided to see whether the synthesis of cytochrome *c* was inhibited by treatment of Krebs cells with ribonuclease. Two batches of cells were exposed to 100μg. of ribonuclease/ml. under slightly differing conditions of osmotic shock, then restored to iso-osmotic buffered glucose and incubated, one with [^{14}C]valine and one with [^{14}C]leucine. As shown in Table 3 there was, in each experiment, strong inhibition of the synthesis of cytochrome *c* and relatively slight or no inhibition of the labelling of total mitochondrial protein.

Moreover, there was close correspondence between the decrease in the synthesis of cytochrome *c* and the synthesis of microsomal and cell-sap proteins. It is concluded that the polypeptide chain of cytochrome *c* must be made on cytoplasmic ribosomes.

Pulse labelling of cytochrome c *in Krebs cells.* Shortly after the addition of a radioactive amino acid to whole cells or after injection into an animal, protein of highest specific radioactivity is found at its site of synthesis, e.g. albumin in liver microsomes

Table 2. *Incorporation of* DL-[^{14}C]*leucine and* DL-[1-^{14}C]*valine into whole protein and into cytochrome* c *during incubation of Krebs cells at* 22° *in Earle's solution*

Cells were incubated aerobically by gently shaking at a cell concentration of 2×10^7/ml. In each experiment 18μc of labelled amino acid was added to 4×10^9 cells. Carrier cells rather than carrier cytochrome *c* was added for extraction of radioactive cytochrome *c* and for determining specific activities of both cytochrome *c* and homogenate protein.

Fraction	Sp. activity of protein (mμc/g.) 30min.	60min.
Expt. 1 Homogenate	118	251
Cytochrome *c* ([^{14}C]leucine-labelled)	72	182
Expt. 2 Homogenate	233	—
Cytochrome *c* ([^{14}C]valine-labelled)	52	—

(Peters, 1962). With Krebs cells it is impossible to obtain sufficient cytochrome *c* from subcellular fractions, especially the microsomal fraction, to determine specific radioactivity, but it was hoped that at early times after adding the labelled amino acid most of the total radioactive cytochrome *c* would be found at its site of synthesis. To determine an appropriate time to fractionate cells to look for the location of the radioactive cytochrome *c* the approximate pool size of incomplete chains of this protein was determined. The technique used was essentially that described by Haldar *et al.* (1967). In two experiments, cells were incubated at 22° with radioactive amino acid for 10 and 30min. respectively; at the end of that time the pool of radioactive amino acid of half of the cells was diluted out by the addition of a large excess of unlabelled carrier and these cells were incubated for a further 20min. Protein synthesis in the other half was stopped quickly by cooling. As already shown, addition of carrier effectively stops further increase in overall radioactivity, but it will not, of course, stop protein synthesis, and incomplete peptide chains will be completed with a concomitant rise in the specific activity of the completed chains. As shown in Table 4, the cytochrome *c* of control cells, in which synthesis was stopped by chilling before addition of unlabelled carrier, was very much less radioactive than the cytochrome *c* of cells subjected to a pulse followed by a chase and further incubation, whereas there was no increase in the radioactivity of total protein after the chase. The increased radioactivity in cytochrome *c* indicates that at 10min. there is a sufficiently large pool of incomplete cytochrome *c* chains and we therefore

Table 3. *Selective inhibition of cytochrome* c *synthesis and of microsomal protein synthesis in Krebs cells subjected to osmotic shock in ribonuclease solution*

A suspension of Krebs cells in Ca^{2+}- and Mg^{2+}-free phosphate saline ($1{\cdot}5 \times 10^8$ cells/ml.) was poured into a solution of ribonuclease in water (100 μg./ml.) at 37°. After the indicated time the solution was rendered iso-osmotic by addition of NaCl–glucose (final glucose concn. 0·01 M). Incubation was continued at 37° and after the indicated time of preincubation 24 μc of [^{14}C]valine (Expt. 1) or [^{14}C]leucine (Expt. 2) was added. After a further 15–20 min. the cells were divided into equal lots. One portion was used for fractionation and the other lot was mixed with carrier cytochrome *c* and the cytochrome *c* reisolated in the usual way. In controls the ribonuclease was omitted. In Expt. 1 cells were 'shocked' for 5 min. and preincubated for 15 min. before addition of [^{14}C]-valine. In Expt. 2 cells were 'shocked' for 7·5 min., preincubated for 12·5 min. and then incubated with [^{14}C]leucine for 20 min.

	Fraction	Ribonuclease (μg./ml.)	Radioactivity (counts/min./mg. of protein)	Percentage change due to ribonuclease
Expt. 1	Homogenate	0	115	
	Homogenate	100	84	−27
	Mitochondria	0	45	
	Mitochondria	100	48	+6
	Microsomes	0	126	
	Microsomes	100	89	−30
	Cell sap	0	103	
	Cell sap	100	83	−20
	Cytochrome *c*	0	136	
	Cytochrome *c*	100	89	−35
Expt. 2	Homogenate	0	472	
	Homogenate	100	252	−45
	Mitochondria	0	160	
	Mitochondria	100	118	−26
	Microsomes	0	641	
	Microsomes	100	277	−57
	Cell sap	0	550	
	Cell sap	100	194	−64
	Cytochrome *c*	0	199	
	Cytochrome *c*	100	52	−73

Table 4. *Completion of cytochrome* c *chains after addition of unlabelled carrier to pulse-labelled Krebs cells*

Each batch of cells (2×10^9) was suspended in Earle's solution (2×10^7 cells/ml.) and incubated for the indicated time at 22° with ^{14}C-labelled amino acid (30 μc in Expt. 1 and 18 μc in Expt. 2). At the end of this time one-half of each batch was removed and cooled to 0°. Unlabelled carrier (80 m-moles/10^8 cells) was added to each batch of cells and the unchilled portion was incubated for a further 20 min. At the end of this time, the cells were spun down and a known amount of carrier mouse-liver cytochrome *c* (10 min. experiment) or non-radioactive cells (30 min. experiment) was added to each batch of cells. Cytochrome *c* was isolated in the usual way (see the Materials and Methods section). The specific radioactivity of a sample of whole cell protein was also determined.

	Radioactivity in protein (mμc/g.)		Percentage increase	
Treatment of cells	Cytochrome *c*	Whole homogenate	Cytochrome *c*	Whole protein
* 10 min.	10·5	741		
* 10 min. + 20 min. chase	24·9	765	137	3
30 min.	51·6	233		
30 min. + 20 min. chase	85·5	217	66	−7

* In this case the specific activity is with respect to cytochrome *c* after addition of carrier and cannot be compared with the specific activity of the protein of the whole homogenate. For the 10 min. experiment we used a mixture of [^{14}C]leucine (15 μc) and [^{14}C]valine (15 μc) and for the 30 min. experiment we used [^{14}C]valine only.

looked for the site in the cell of radioactive cytochrome *c* at this time.

Location of labelled cytochrome c *in subcellular fractions.* Cells were labelled for 10 min. at 22°, cooled and disrupted in sucrose and the subcellular fractions were separated by differential centrifuging. Cytochrome *c* was isolated from each fraction as described in the Material and Methods

Table 5. *Localization of radioactive cytochrome* c *in Krebs cells after incubation of cell suspensions with* [^{14}C]*leucine*

A suspension of 4×10^9 cells in Earle's solution was incubated for 10 min. at 22° with 40 μC of DL-[1-^{14}C]leucine. The cells were divided into two equal portions and one portion was washed with 0·3 M-sucrose and disrupted with an Ultra-Turrax disintegrator as described by Freeman (1965). From the other portion the nuclear, mitochondrial, microsomal and cell-sap fractions were separated as described by Haldar *et al.* (1967) and the nuclei and mitochondria were washed once with sucrose–EDTA–nicotinamide (Freeman, 1965). About 10^9 unlabelled cells were added to each subcellular fraction, cytochrome *c* was extracted and measured and a further 2·0 mg. of carrier mouse-liver cytochrome *c* was added for further purification.

	Fraction	Radioactivity recovered in cytochrome *c* ($\mu\mu$C)
Expt. 1	Whole cells	218
	Nuclei	12
	Mitochondria	106
	Microsomes	22
	Cell sap	31
	Recovered in fractions	171
Expt. 2	Whole cells	340
	Nuclei	33
	Mitochondria	232
	Microsomes	25
	Cell sap	95
	Recovered in fractions	385

section and Table 5. In this way the total radioactivity of the reisolated cytochrome *c* was a measure of the amount of radioactive cytochrome *c* in each fraction. The results are given in Table 5. The total recovered radioactive cytochrome *c* in the fractions was about that of the whole cells and indicates that there was no serious loss resulting from fractionation. Allowing for the difficulty in purifying small amounts of cytochrome *c* it is reasonably clear that nearly all the radioactive cytochrome *c* was located in the mitochondria after a 10 min. pulse of labelling at 22°.

DISCUSSION

Evidence has already been presented (Haldar *et al.* 1966, 1967) that there are two independent sites of protein synthesis in mammalian cells, the mitochondria and the cytoplasmic ribosomes. It was found, however, that when mitochondria were incubated with radioactive amino acids *in vitro* only some of the mitochondrial proteins became labelled and both malate dehydrogenase and cytochrome *c* were unlabelled. It seemed therefore that we had to consider three possible explanations: (*a*) cytochrome *c* and other soluble mitochondrial proteins are synthesized on cytoplasmic ribosomes but transferred after synthesis to the mitochondria; or (*b*) two types of protein synthesis occur in mitochondria, only one of which survives during incubation of isolated mitochondria; or (*c*) the labelling observed *in vitro* is an artifact and has no biological significance. We have already given our reasons and our evidence for believing that explanation (*c*) can be eliminated (Roodyn, 1965, 1966; Haldar *et al.* 1966, 1967), and the present investigation has been devoted to distinguishing between the two possibilities (*a*) and (*b*).

Penetration of ribonuclease into Krebs cells selectively inhibits both microsomal protein synthesis (Haldar *et al.* 1967) and cytochrome *c* synthesis to a comparable extent (Table 3), while leaving the synthesis of total mitochondrial protein relatively unaffected. It is clear from this result that the peptide chain of cytochrome *c* is synthesized on cytoplasmic ribosomes.

There remains the slightly puzzling fact that even during pulse labelling much more radioactive cytochrome *c* is found in the mitochondrial fraction than in the microsomal fraction. One possibility is that the cytochrome *c* does not assume its final conformation until after it has passed into mitochondria.

Mitochondria are known to be a major site for synthesis of porphyrins and, moreover, the enzyme that inserts iron into porphyrins is thought to be located entirely within mitochondria (Reithmüller & Tuppy, 1964; Sugimura, Okabe, Nagao & Gunge, 1966). It is a reasonable supposition therefore that the peptide chain of cytochrome *c* is not attached to its haem residue until it has passed from the microsomes to the mitochondria. Although this is plausible it is equally likely that either of the following explanations could account for the result. Cytochrome *c* could be completely synthesized on cytoplasmic ribosomes, but it could not be detected because there is only a small rapidly turning-over pool present at its site of synthesis. If specific activities of cytochrome *c* of subcellular fractions could have been determined this difficulty would be overcome. Alternatively, cytochrome *c* could be passed directly into mitochondria as it is being made. This would be comparable with the vectorial discharge of proteins from ribosomes to the membrane of the endoplasmic reticulum in liver (Redman & Sabatani, 1966). Clearly, further information is needed to clarify this point.

Although there are obvious limitations in the validity of comparisons of such widely differing cells as the Krebs cells and *Saccharomyces cerevisiae* it is noteworthy that isolated yeast mitochondria will synthesize protein under similar conditions to

Krebs-cell mitochondria (Wintersberger, 1965), and in these experiments also there was little or no labelling of the readily soluble proteins. Available genetic evidence suggests that the genes controlling cytochrome *c* synthesis are true nuclear genes (Sherman, Stewart, Margoliash, Parker & Campbell, 1966). This in itself does not, of course, eliminate mitochondria as a possible source of synthesis, since messenger RNA could presumably reach mitochondria from the nuclear genes, but the evidence now presented is most readily understood if it is assumed that the messenger RNA of the nuclear genes functions entirely through the microsomal ribosomes and that mitochondrial DNA provides the genetic information required for synthesis within mitochondria.

Mitochondria therefore do not have the character of self-replicating entities. They may in fact undergo binary fission (Luck, 1963), but they certainly cannot be regarded as cells within cells. Some understanding of the processes involved in replication of mitochondria has begun to emerge and the results so far serve to emphasize the importance of hydrophobic proteins (structural proteins) in ordering the morphology of intracellular structure.

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REFERENCES

Chance, B. & Hess, B. (1959). *J. biol. Chem.* **234**, 2404.

Craig, L. C. (1960). In *A Laboratory Manual of Analytical Methods of Protein Chemistry (including Polypeptides)*, vol. 1, p. 103. Ed. by Alexander, P. & Block, R. J. Oxford: Pergamon Press Ltd.

Freeman, K. B. (1965). *Biochem. J.* **94**, 494.

Haldar, D., Freeman, K. B. & Work, T. S. (1966). *Nature, Lond.*, **211**, 9.

Haldar, D., Freeman, K. B. & Work, T. S. (1967). *Biochem. J.* **102**, 684.

Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc.* B, **122**, 298.

Luck, D. J. L. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 233.

Margoliash, E. & Frohwirt, N. (1959). *Biochem. J.* **71**, 570.

Margoliash, E. & Lustgarten, J. (1962). *J. biol. Chem.* **237**, 3397.

Margoliash, E., Smith, E. L., Kreil, G. & Tuppy, H. (1961). *Nature, Lond.*, **192**, 1121.

Martin, E. M., Malec, J., Sved, S. & Work, T. S. (1961). *Biochem. J.* **80**, 585.

Paléus, S. & Theorell, H. (1957). *Acta chem. scand.* **11**, 905.

Paul, K. G. (1955). In *Methods in Enzymology*, vol. 2, p. 749. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Peters, T. (1962). *J. biol. Chem.* **237**, 1181.

Redman, C. M. & Sabatani, D. D. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 608.

Reithmüller, G. & Tuppy, H. (1964). *Biochem. Z.* **340**, 413.

Roodyn, D. B. (1965). *Biochem. J.* **97**, 782.

Roodyn, D. B. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 562. Ed. by Trager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.

Roodyn, D. B., Suttie, J. W. & Work, T. S. (1962). *Biochem. J.* **83**, 29.

Rosenthal, O. & Drabkin, D. L. (1943). *J. biol. Chem.* **149**, 437.

Sandell, S., Löw, H. & Decken, A. von der (1966). *Abstr. 3rd Meet. Europ. Fed. biochem. Soc., Warsaw*, p. 167.

Sherman, F., Stewart, J. W., Margoliash, E., Parker, J. & Campbell, W. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 1498.

Smithies, O. (1955). *Biochem. J.* **61**, 624.

Steinberg, D. (1960). *Analyt. Biochem.* **1**, 23.

Sugimura, T., Okabe, K., Nagao, M. & Gunge, N. (1966). *Biochim. biophys. Acta*, **115**, 267.

Suttie, J. W. (1962). *Biochem. J.* **84**, 382.

Van Gelder, B. F. & Slater, E. C. (1962). *Biochim. biophys. Acta*, **58**, 593.

Wintersberger, E. (1965). *Biochem. Z.* **341**, 409.