Review Letter

MITOCHONDRIAL BIOGENESIS: GERM-FREE MITOCHONDRIA

D. B. RODOYN

Department of Biochemistry, University College London, London W. C. 1, U. K.

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Biologists have long been interested in the problem of the origin and mechanism of replication of the mitochondrion, and the early views are admirably discussed in Lehninger's book 'The Mitochondrion' (Benjamin Press, 1964). The last few years have seen a dramatic increase in the research on the subject and at the recent FEBS meeting in Prague Dr. Lars Ernster showed that research papers in this topic were now in the early logarithmic phase of increase! There are now many detailed reviews on the subject, and at least two books exclusively devoted to the topic (Roodyn & Wilkie: 'The Biogenesis of Mitochondria', Methuen Press, 1968, and 'Biochemical Aspects of the Biogenesis of Mitochondria' edited by Slater, Tager, Papa and Quagliariello; Adriatica Editrice, Bari, 1968). The second of the books includes some detailed discussion about the problem of bacterial contamination in studies of the biosynthetic activity of isolated mitochondria, in particular the incorporation of radioactive amino acids *in vitro*. Since there has been a certain amount of confusion in the literature about the matter, this is perhaps a useful occasion to report on the present situation.

It has been known for many years that rat-liver mitochondria prepared under non-sterile conditions contain a variable number of different bacteria, ranging from 1000 to 100 000 organisms/ml (see for example Roodyn, Reis & Work, Biochem. J. 80 (1961) 9). Such mitochondrial fractions also incorporate radioactive amino acids into their protein. The incorporation does not require cell sap or pH 5 enzymes, and can be observed in relatively simple media that would be favourable for bacterial contaminants. The incorporation is not inhibited by ribonuclease and it is also sensitive to many antibacterial antibiotics, in particular chloramphenicol. Lamb, Clark-Walker & Linnane (Biochim. biophys. Acta 161 (1968) 415) have just published a detailed study of the effect of antibiotics on yeast mitochondria and cytoplasmic ribosomes. The mitochondria are inhibited by a range of antibacterial antibiotics but are unaffected by cycloheximide, whereas the converse is true for the cytoplasmic ribosomes.

It is not surprizing, therefore, that people working on such systems should have anxieties about possible bacterial contamination. During the investigation of the general properties of the mitochondrial system, however, it soon appeared from indirect evidence that it was unlikely that bacterial contamination was a serious source of error. The incorporation occurred in a complex medium with succinate as an energy source, but not if the succinate was replaced by glucose. It was dependent on added P_i, Mg^{2+} ions and adenine nucleotides. It was sensitive to mechanical damage of the mitochondria and to the tonicity of the isolation medium. There was no obvious correlation with bacterial counts, and the labelled particles co-sedimented precisely with mitochondria and not with bacteria in sucrose gradients. It was found that treatment of thyroidectomized rats with thyroid hormone resulted in a stimulation in incorporation about two days later, an effect that was very hard to explain in terms of bacterial contamination. The system also responded to various physiological changes in the whole animal (e.g. was greater in young than in old animals). Incorporation ceased after 1-1½ hr and if anything the specific activity of the radioactive protein was less after 3 hr than after 2 hr incubation. These arguments are summarized in pp. 562-563 of 'Regulation of Metabolic Processes in

Mitochondria' edited by Tager et al., Elsevier Press (1966).

In 1965, however, der Decken, Sandell & Löw questioned the significance of incorporation studies with isolated mitochondria (pp. 415-425 of 'Regulation of Metabolic Processes in Mitochondria'). They found that if sub-mitochondrial particles were passed through a millipore filter they no longer incorporated amino acids *in vitro*. Also, intact liver mitochondria that had only 100 bacteria/ml had negligible activity. In a more extensive study Sandell, Löw & der Decken (Biochem. J. 104 (1967) 575) repeated their observations that germ-free mitochondria were inactive *in vitro*. They also made the interesting observation that the incorporation activity of dilute bacterial suspensions *in vitro* was stimulated by the addition of inactive germ-free mitochondria. Although these authors suggested that the incorporation they observed with non-sterile mitochondria was due to bacteria, in fact the 'reconstructed' systems of bacteria plus mitochondria behaved differently to the usual mitochondrial system in that the incorporation curve appeared to be exponential. Nevertheless the stimulation of bacterial activity by added mitochondria was a disturbing observation. Beattie, Basford & Koritz (J. Biol. Chem. 242 (1967) 3366) then reported that they were able to demonstrate incorporation activity in germ-free mitochondria. However, they confirmed that mixtures of bacteria and inactive mitochondria gave measurable radioactivity *in vitro* and questioned the validity of work that had been done with non-sterile mitochondria. Wheeldon (Biochem. Biophys. Res. Commun. 24 (1966) 407) was able to separate mitochondrial membranes from bacteria, and concluded that although part of the incorporation observed *in vitro* was due to mitochondria, the ribonuclease resistant incorporation was due to bacteria.

For a time therefore there was a somewhat contradictory situation. A great body of indirect evidence suggested that the incorporation observed with non-sterile mitochondrial fractions could not have been due to bacteria. However, those direct experiments aimed at elucidating the matter seemed to show that contamination was an important factor. Fortunately a number of further studies in the last two years have greatly clarified the situation. In addition to the paper of Beattie and co-workers mentioned above, there are now several reports of active amino acid incorporation by germ-free mitochondria. Yellin, Butler & Stein (Fed. Proc. 26 (1967) 833) obtained amino acid incorporation with sterile rat-brain mitochondria, and Lado & Schwendimann (It. J. Biochem. 15 (1967) 279) found that germ-free plant mitochondria incorporated *in vitro*. On p. 369 of the 'Biochemical Aspects of the Biogenesis of Mitochondria' (see above) Work describes the isolation of germ-free mitochondria from embryonic rat-liver. The embryos were removed by Caesarean sections under sterile conditions, and the final mitochondrial fractions were fully active in amino acid incorporation but contained no detectable micro-organisms. Grivell (Biochem. J. 105 (1967) 44c) developed a procedure for isolating yeast mitochondria that contained very low numbers of contaminating micro-organisms, and found that they were able to incorporate radioactive amino acids *in vitro* quite actively. The incorporation was inhibited by chloramphenicol, but in contrast to the observations of Wheeldon mentioned above, the germ-free system was quite resistant to ribonuclease. We have recently analysed the labelled yeast mitochondrial fraction on sucrose gradients, monitoring the particles with the aid of an Autoanalyzer (Roodyn & Grivell, FEBS Letters 1 (1968) 166). The radioactive particles co-sediment precisely with mitochondria as measured by a succinate dehydrogenase marker. These results thus fully confirm the findings of Wintersberger (Biochem. Z. 341 (1965) 409) that yeast mitochondria incorporate amino acids *in vitro*.

There have also been some comparative studies with sterile and non-sterile mitochondria that seem to resolve the problem completely. Kroon, Saccone & Botman (Biochim. Biophys. Acta 142 (1967) 552) found that rat-liver mitochondria isolated under sterile conditions had precisely the same incorporation activity *in vitro* as non-sterile mitochondria. In addition, the incorporation was ribonuclease resistant, again in contrast to Wheeldon's observations. In similar experiments Fournier & Simpson (on pp. 241-244 of 'Biochemical Aspects of the Biogenesis of Mitocondria') describe the preparation of mitochondria from germ-free rats. They found that preparations that contained only 40 organisms/ml at the beginning of the experiment had exactly the same incorporation activity as non-sterile preparations with about 10^5 bacteria/ml. They also showed that if non-sterile mitochondria were incubated with radioactive amino acid

and analysed on a sucrose gradient, it was possible to obtain a 'bacterial band' containing about 70% of the total bacteria, completely free of mitochondria. These bacteria were found to have no detectable radioactivity. It therefore appears to be the happy circumstance that up to 100 000 contaminant bacteria/ml produce absolutely no interference in the system. This, of course, is in excellent agreement with the many indirect arguments against bacterial contamination cited above.

How can we reconcile these findings with the experiments of Sandell & co-workers and Beattie & co-workers? It is of course possible that 10^5 organisms, even if fully active, would not give a measurable radioactivity. Lamborg & Zamecnik (Biochim. Biophys. Acta 42 (1960) 206) found that at least 100 000 *E.coli* cells are required to give measurable counts *in vitro*. However, this ignores the important observation that the bacterial incorporation is much greater in the presence of mitochondrial fractions, which possibly add co-factors that stimulate the bacterial system. These effects, however, had been observed with bacteria that had been cultured on various media before the experiment. The most probable explanation for the apparent contradiction in results is that the bacteria that happen to contaminate mitochondrial fractions are in a very poor functional state and hence cause no interference at least for several hours. Kroon, Botman & Saccone discuss the matter at length on pp. 439-449 of 'Biochemical Aspects of the Biogenesis of Mitochondria'. They observed a mixed population of bacteria and moulds in their non-sterile mitochondria, ranging from 5000 to 100 000 cells/mg protein. However, these cells did not increase significantly in numbers during the course of 2 hr incubation. Also, four strains of bacteria isolated from the mitochondrial fraction grew poorly in the mitochondrial incorporation medium. Further, if these bacteria were left at 2° for about a week they lost a great deal of their incorporation activity. In the Discussion, Kroon pointed out that it is most likely that much of the contamination comes from the skin and hair of the animals, and in this case one would expect the bacteria to be in a poor metabolic condition.

There now seems little doubt, therefore, that isolated mitochondria are fully able to incorporate radioactive amino acids *in vitro* and also that the results observed with active non-sterile mitochondrial fractions were not, in general, due to the variable numbers of bacteria present. Beattie, Basford & Koritz (Biochem. 6 (1967) 3099) have shown that the properties of the sterile system are essentially the same as those observed by other workers with non-sterile preparations. In particular, the major radioactive products are insoluble proteins in the inner mitochondrial membrane (see Sebald, Bücher, Olbrich & Kaudewitz, FEBS Letters 1 (1968) 235, for recent experiments on the radioactive products). The problem of bacterial contamination does not only arise in studies on mitochondrial protein synthesis, however. Experiments with DNA and RNA synthesis, ribosomes, tRNA and activating enzymes could all be subject to interference. Fortunately the successful development of techniques for the isolation of mitochondria that contain negligible numbers of micro-organisms means that one need no longer rely on indirect arguments, however convincing they appear to be. Since many striking similarities have recently been found between mitochondrial and bacterial biosynthetic systems (see 'The Biogenesis of Mitochondria') it is essential that the validity of any future experiments should not be questioned by the spectre of bacterial contamination. The controversy over the possible role of bacterial contaminants in experiments on mitochondrial protein synthesis has therefore been a most useful stimulus to the development of more rigorous techniques for the study of mitochondrial biogenesis.