The protein-synthetic system of rat-liver mitochondria. Its characterization and its response to inhibitors
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4.1. SUMMARY

This thesis deals with the protein-synthetic system of rat-liver mitochondria. Two aspects were studied: (1) the isolation and characterization of mitochondrial ribosomes (Papers III and IV); (2) the effect of inhibitors of translation or transcription on mitochondrial protein synthesis both in vivo (Papers I and II) and in vitro (Papers III–V).

It was shown that rat-liver mitochondrial ribosomes have a sedimentation coefficient of about 55S and consist of 39S and 28S subunits. The RNA from the large subunit is about 19S, the RNA from the small subunit is about 15S. The migration behaviour of the 55-S ribosomes on 0.4% agarose gels showed that the charge/mass ratio is low as compared to rat-liver cytoplasmic and to bacterial ribosomes. This is in accordance with a RNA content of only about 37%, calculated from the buoyant density in CsCl. From electrophoresis of the ribosomes in gels containing a gradient of polyacrylamide it was concluded that the volume of 55-S ribosomes is intermediate between those of cytoplasmic 80-S and bacterial 70-S ribosomes. These data together, in combination with the molecular weight of the ribosomal RNAs, strongly suggest that the 55-S ribosome has a mass about equal to that of bacterial ribosomes, and an even larger volume. Results obtained independently in other laboratories also provide evidence that the 55-S ribosome is not a "mini-ribosome", in spite of its low S-value and small RNAs.

The mitochondrial ribosomes were also characterized functionally. After incubation of isolated mitochondria with [3H]-leucine labelled nascent peptides were found to sediment at 57-S in an isokinetic sucrose gradient. Puromycin released these peptides, showing the ribosomal nature of the particles, and chloramphenicol inhibited the synthesis of these peptides, showing the mitochondrial nature of the ribosomes. Isolated 55-S ribosomes were further characterized by means of assaying peptidyl transferase activity by a modified "fragment reaction". It was shown that a chloramphenicol-sensitive peptidyl transferase activity was present in the 55-S ribosome and in its large subunit. An aspecific side reaction occurring in the absence of puromycin and leading to high blanks in the "fragment reaction" could be tracked down to the contamination of ribosomal fractions with lysosomal hydrolytic enzymes: after treatment of the mitochondria with digitonin to remove lysosomes the blanks without puromycin dropped to nearly zero. This digitonin treatment solved also another problem: by the treatment an 82-S fraction, hitherto always present in our ribosomal preparations, almost completely disappeared. It therefore seems probable that this 82-S fraction contained in majority cytoplasmic ribosomes adhering to the mitochondrial outer membrane, and a small amount of 55-S ribosomes, present either as cross-contamination or as dimers.

Finally, the capacity of the 55-S ribosomes to perform poly(U)
directed polyphenylalanine synthesis was investigated. Although we found activity in this reaction, the absolute activity was very low (25 pmole/mg ribosomal RNA per 15 min). Other investigators, however, found a much higher activity of rat-liver mitochondrial 55-S ribosomes in this system. In view of these data it can be concluded that the 55-S particle is the native rat-liver mitochondrial ribosome.

Another part of this thesis deals with the effect of chloramphenicol and oxytetracycline, and of ethidium bromide and euflavin on the \textit{in vivo} formation of cytochrome $c$ oxidase (cytochrome $aa_3$). These four drugs strongly inhibited the formation of this mitochondrial enzyme in regenerating rat liver, without affecting liver regeneration itself. Regarding chloramphenicol and oxytetracycline, both inhibitors of bacterial and mitochondrial protein synthesis, these results show that the action of these drugs is the same in animals as in yeast and that the mitochondrial protein-synthesizing system is involved in the formation of functionally active and spectrally recognizable cytochrome $c$ oxidase. Indeed, recent findings in other laboratories showed that in \textit{Neurospora} and in yeast 3 out of 7 peptide components of the enzyme are synthesized within the mitochondria. In striking contrast to the results with chloramphenicol and oxytetracycline, the inhibition by ethidium bromide and euflavin, both inhibitors of mitochondrial transcription by intercalation into mitochondrial DNA, seemed irreversible: only one injection of a low dose of either drug inhibited cytochrome $c$ oxidase formation for at least two days. Moreover, we found earlier that cytochrome $c$ oxidase formation in cultured heart cells remained blocked for at least 6 days after removal of these drugs. Since a direct interaction of the dyes with translation is unlikely in view of the absence of inhibition in bacterial and microsomal cell-free protein synthesis, it was concluded that formation of cytochrome $c$ oxidase is dependent not only on mitochondrial translation but also on mitochondrial transcription. The irreversibility of the inhibition led us to conclude that the mitochondrial DNA had become persistently altered by the action of the dyes. In view of later results obtained by other authors as well as in our laboratory it now appears more likely that this irreversibility, at least in the heart cells, reflects a persisting presence of the drugs in the mitochondria rather than an alteration of the DNA.

Finally, the influence of macrolide antibiotics on the mitochondrial protein synthetic system was investigated. Tylosin tartrate, spiramycin and carboxymycin strongly inhibited protein synthesis by isolated rat-liver or BHK-21 mitochondria, whereas erythromycin inhibited BHK-21 mitochondria but not intact rat-liver mitochondria. Furthermore, it was found earlier that swollen rat-liver mitochondria are inhibited by erythromycin. Thus, it appears that mammalian mitochondrial ribosomes themselves are sensitive to erythromycin, but that the mitochondrial membranes are impermeable to the drug. Consequently, we investigated the response of isolated 55-S ribosomes and of \textit{Neurospora crassa} mitochondrial ribosomes to the macroli-
des in the "fragment reaction". Tylosin tartrate, spiramycin and carbomycin inhibited this reaction, whereas erythromycin could reverse the chloramphenicol inhibition (erythromycin has no direct effect on peptidyl transferase, but can reverse the inhibition by chloramphenicol). For both N. crassa and rat-liver mitochondrial ribosomes rather high concentrations (200 µg/ml or higher) were necessary to obtain inhibition or, for erythromycin, reversal of chloramphenicol inhibition. Earlier results by us showed that yeast mitochondrial ribosomes are relieved from chloramphenicol inhibition by low erythromycin concentrations (10 µg/ml). For carbomycin evidence is given that mitochondria concentrate the drug 30-fold or more from the medium. This might explain, at least in part, that isolated mitochondria are much more sensitive to carbomycin than the isolated 55-S ribosomes. From these results we conclude that mitochondrial ribosomes both from animals and from Ascomycetes are sensitive to macrolides, but that animal mitochondrial membranes may withhold some from entering the mitochondria. Furthermore, significant species variations in sensitivity occur, even among mitochondrial ribosomes from yeast and from N. crassa, both Ascomycetes. Therefore, in spite of the heterogeneity in physical properties among mitochondrial ribosomes and the differences with bacterial ribosomes, a remarkable uniformity is maintained in their response to antibiotics inhibiting protein synthesis.

4.2. SAMENVATTING.

Het onderwerp van dit proefschrift is het eiwitsynthetiserend systeem van rattelevermitochondriën. Twee aspecten werden bestudeerd: (1) de isolering en karakterisering van mitochondriale ribosomen (artikelen III en IV); (2) het effect van remmers van translatie en transcriptie op de mitochondriale eiwitsynthese, zowel in vivo (artikelen I en II) als in vitro (artikelen III-V).

Het bleek dat mitochondriale ribosomen uit rattelever een sedimentatiecoëfficiënt van ca. 55S hebben en bestaan uit subeenheden van 39S en 28S. Het RNA van de grote subeenheid is ongeveer 19S, dat van de kleine subeenheid ongeveer 15S. Het migratiegedrag van 55S ribosomen op 0,4 % agarose gels wees erop dat de lading/massa verhouding laag is vergeleken met cytoplasmatische ribosomen uit rattelever en met bacteriële ribosomen. Dit kan worden verklaard met het RNA-gehalte van slechts 37 %, berekend uit de zweefdichtheid in CsCl. Het electroforesegredrag in gels met een polyacrylaamde-gradiënt toonde aan dat het volume van 55S ribosomen ligt tussen dat van cytoplasmatische 80S en bacteriële 70S ribosomen. Tezamen met het moleculair gewicht der ribosomale RNA's suggereren deze gegevens sterk, dat het 55S ribosoom een massa heeft die ongeveer gelijk is aan die van bacteriële ribosomen, en zelfs een groter volume. Ook resultaten uit andere laboratoria geven aan dat het 55S ribosoom, ondanks zijn large S-waarde en kleine RNA's, geen "mini-ribosoom" is.