# STUDIES ON NUCLEOTIDE INCORPORATION INTO MITOCHONDRIAL RNA

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Data are accumulating suggesting the occurence of DNA in mitochondria (1-4). In addition, actinomycin-sensitivity of amino acid incorporation into mitochondrial protein (5,6), as well as of nucleotide incorporation into mitochondrial acid-precipitable material (7,6), has been demonstrated in different mitochondrial preparations. Actinomycin is thought to represent a rather specific inhibitor of the DNA-dependent RNA synthesis (8-10).

The above findings suggest the possibility that a DNA-dependent RNA synthesis also occurs in mitochondria. So far such a process has been proven to exist only in cell nuclei (11). In this paper RNA synthesis in mitochondria is demonstrated and some characteristics of this process are given.

EXPERIMENTAL - To exclude the interference of nuclear polymerase possibly contaminating the preparations, special care was taken to obtain mitochondrial fractions as pure as possible, accepting large losses of the particles during the isolation procedure. For each of the experiments about 20 rat livers were used (female Sprague-Dawley). The low-spin centrifugation of the homogenate (0,25 M sucrose) effecting the removal of liver nuclei and intact cells (usually about 600 x g) was raised to about 1000 x g and repeated 2-3 times. The mitochondria were washed at least four times. The purity of the individual fractions was controlled with the phase contrast microscope during the isolation process. In many cases additional electron microscopic pictures were taken. These also showed the final preparations to be essentially free of intact nuclei.
The cell particles were incubated in the presence of the labeled nucleotides and the RNA was extracted using a procedure based on the method of OGUR and ROSEN (12) or isolated with phenol according to KIRBY (13). Radioactivity incorporated was measured in a Packard automatic TRI-CARB liquid scintillation counting system (314 EX). All samples were counted 3-6 times for 30 minutes.

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RESULTS.- A. Experiments with "intact" mitochondria.

Table I,A, examplifying a typical experiment, shows the mitochondrial fraction as being capable of incorporating $C^{14}$-UTP into RNA. Using an incubation time of 5 minutes and 1,0 µC of labeled UTP per test sample the incorporation rate was low but significant. By adding $H^3$-UTP of high specific activity (3,2 mC/µmol) instead of $C^{14}$-UTP (19 - 25 µC/µmol) to the medium the radioactivity of the isolated RNA could be increased 5-10 fold.

Table I. UTP-$C^{14}$-incorporation into "intact" rat liver mitochondria.

| | | c.p.m. | c.p.m. /10µg RNA |
|---|---|---|---|
| Mitochondria, A. | complete | 16 | 11 |
| | + 40 µg/ml RNAase | 16 | 12 |
| | + 12 µg/ml Actinomycin $C_1$ | 15 | 10 |
| | + 7 mM Pyrophosphate | 1* | 1 |
| | CTP omitted | 14 | 10 |
| Nuclei , B. | complete | 23 | 100 |
| | + 40 µg/ml RNAase | 2* | |
| | + 12 µg/ml Actinomycin $C_1$ | 1* | |
| | + 7 mM Pyrophosphate | 0* | |
| | CTP omitted | 2* | |
| 0-time | | 2 ± 2 | |

* not significant above the background or the 0-time blank.

After incubation for 5 minutes at 37° the reaction was terminated by 2% perchloric acid. Data are given for 1/10 of the total amount extracted, and are corrected for background (42 ± 3 c.p.m. = extreme limits when counting more than hundred samples; the background represents the combined counting error and the variance of the apparatus).
10 mg mitochondrial protein were used per assay.
The assay system contained : 0,3 ml of the cell fractions, 1,0µC 2-$C^{14}$-UTP (19 µC/µmole), 50 mM KCl, 5 mM $MgCl_2$, 0,5 mM EDTA, 4 mM GSH, 0,4 mM ATP and GTP (also CTP, if present), 16 mM phosphate, 100 mM Tris-HCl, pH 7,4, 4 mM Phosphoenolpyruvate, and 0,01 ml pyruvate kinase, BOEHRINGER (10 mg/ml).

Amino acid incorporation into mitochondrial protein has been shown to be insensitive to RNAase (14). Similarly, the nucleotide incorporation into these particles was very slightly affected by RNAase and actinomycin $C_1$. The addition of other nucleotide triphosphates besides the labeled one was not necessary in order to demonstrate the incorporation. These results may exclude a contamination of the mitochondrial fractions with RNA polymerase from nuclear fragments as a source of incorporating activity, because the $C^{14}$-UTP incorporation into isolated

nuclei is highly sensitive to RNAase and actinomycin. Table I B gives an example performed with an amount of rat liver nuclei catalyzing an incorporation comparable to that of mitochondria. Furthermore, the nuclear process does not proceed without the addition of other nucleotide triphosphates (ATP, GTP, CTP).

$C^{14}$-UTP incorporation into mitochondrial RNA could not only be demonstrated with rat liver preparations but also with mitochondrial fractions from other tissues, such as pigeon heart, pigeon breast muscle and some tumors. Pigeon heart mitochondria seem to be especially suitable, since high incorporation rates were obtained with preparations showing a comparatively low RNA content.

Incubation in the presence of RNAase repeatedly caused a marked decrease of the RNA content in "intact" mitochondria, without affecting the incorporation rate of $C^{14}$-UTP. Thus, a distinction between two different types of mitochondrial RNA seems probable.

B. Experiments with swollen mitochondria.

The insensitivity of the mitochondrial system to RNAase and actinomycin was suspected to be due to the impermeability of the mitochondrial membrane to these large molecules. At the same time, "intact" mitochondria apparently contain a high enough concentration of the four nucleotide triphosphates necessary to allow for the incorporating reaction. These assumptions were tested by increasing the permeability of the mitochondrial membrane. This was achieved by a number of procedures including repeated washings in sucrose without EDTA, or preincubating the particles with inorganic phosphate in the absence of respiratory substrate, or by a brief treatment with hypotonic solutions. Mitochondria treated in one of these ways retained the capacity of incorporating labeled UTP into RNA. However, in contrast to the earlier mentioned "intact" particles, the UTP-incorporating process in these partly damaged, nucleotide-depleted preparations depended strictly on the presence of the other three nucleotide triphosphates, as shown for CTP (Table II). In addition, under these experimental conditions the reaction was completely inhibited or considerably diminished by actinomycin $C_1$ and partly sensitive to high concentrations of RNAase. Furthermore, an inhibition by DNAase could be demonstrated in these altered mitochondrial preparations, especially with pigeon heart. Since the RNA content of the particles did not change during this DNAase action, an inhibition by the RNAase contaminating the DNAase preparation used could be excluded (table II).

Table II. UTP-incorporation into rat liver mitochondria with altered permeability.

| | Label | c.p.m. | µg RNA | c.p.m. /10µg RNA |
|---|---|---|---|---|
| I. complete | $C^{14}$ | 27 | 21 | 13 |
| + 12 µg/ml Actinomycin $C_1$ | | 4 | 22 | 2 |
| + 40 µg/ml RNAase | | 16 | 16 | 10 |
| + 80 µg/ml DNAase | | 19 | 21 | 9 |
| + hexokinase-glucose | | 7 | 21 | 3 |
| CTP omitted | | 5 | 20 | 3 |
| + microsomes | | 25 | 35 | 7 |
| II. complete | $H^3$ | 149 | 27 | 55 |
| + 12 µg/ml Actinomycin $C_1$ | | 62 | 27 | 23 |

Rat liver mitochondria preincubated with inorganic phosphate in the absence of respiratory substrate (10 minutes, 25°). Experimental conditions as described in table I. 2,0 µC 2-$C^{14}$-UTP (25 µC/µmole) or 50 µC $H^3$-UTP (3,2 mC/µmole) were used. Incubation time 5 minutes at 37°. The cpm-values are corrected for background (43 ± 2 cpm). The percentage of inhibition by actinomycin was found between 65 and 100% in 5 additional experiments performed under the same conditions. It is seen that a moderate contamination of the mitochondrial preparation with microsomes will not influence the absolute incorporation rate, but of course the specific activity.

Radioactivity was also found in the RNA when $C^{14}$-UTP was replaced by $C^{14}$-GTP or $C^{14}$-ATP, indicating that the other nucleotide triphosphates were incorporated in a similar manner as UTP.

Under special conditions in a few experiments an actinomycin-insensitive nucleotide incorporation was observed which was exceptionally sensitive to RNAase. This phenomenon might represent either an exchange reaction or a coupling of the mononucleotide to terminal positions of the polynucleotide chains. It is under further investigation..

The data indicate that mitochondria contain the enzymic apparatus for incorporating UTP and other suitable nucleotide triphosphates into RNA. Sensitivity of such a process to actinomycin is commonly regarded as evidence for a DNA dependency. We do not consider an effect of a single inhibitor to be sufficient evidence, especially in the case of

actinomycin which might have actions besides those known to occur at the DNA level (15). But actinomycin sensitivity together with the finding of an inhibition produced by DNAase favours the idea of a DNA participation in the process investigated.

This conclusion is given additional support by our studies on amino acid incorporation into mitochondrial protein. This process shows some of the characteristics of protein synthesis, like RNAase sensitivity and the necessity for the presence of other amino acids besides the labeled one (16). A stimulation of this reaction is possible by adding a fourth nucleotide (CTP) to an assay system supplemented with the other three nucleotide triphosphates (ATP, GTP, UTP (17). Furthermore, a DNAase sensitivity to a degree comparable to the effect of actinomycin could be demonstrated in suitable preparations.

Although final evidence for the mitochondrial origin of DNA extracted from such preparations is still lacking *) the data presented suggest the existence of a functional DNA within the mitochondria which could form the basis for a "cytoplasmic heredity", as postulated as early as 1908 (18). Further studies are in progress to explore the possible implications of these findings for tumor metabolism and the action of toxins.

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Bibliography

(1) CHEVREMONT,N: Biochem.J. 85, 25 p (1962)
(2) NASS,M.M.K. and NASS,S.: J.Cell Biol. 19, 593, 613 (1963)
(3) GUTTES,E. and GUTTES,S.: Science 145, 1057 (1964)
(4) SCHATZ,G., HASLBRUNNER,E.und TUPPY,H.: Biochem.biophys.Res.Commun. 15, 127 (1964)
(5) KROON,A.M.: Biochim.biophys.Acta 76, 165 (1963)
(6) NEUBERT,D., TIMMLER,R. und HELGE,H.: Arch.exp.Path.u.Pharm.in press
(7) WINTERSBERGER,E.: Z.physiol.Chem. 336, 285 (1964)
(8) REICH,E., FRANKLIN,R.M. und SHATKIN,A.J.and TATUM,E.L.: Science 134, 556 (1961)

*) The mitochondrial preparations used in this study contained extremely small amounts of DNA - at least one order of magnitude less than reported for yeast mitochondria (4).

Addendum. Since this manuscript was prepared the recent reports of Luck and Reich (Proc. Nat. Acad. Sci., U.S. 52, 931 (1964) and Kalf (Biochemistry, 3, 1702 (1964)) came to the authors' attention. They describe the occurrence of a DNA-dependent RNA polymerase in mitochondria of Neurospora crassa and beef heart respectively.

(9) GOLDBERG,I.H. and RABINOWITZ,M.: Science 136, 315 (1962)
(10) HURWITZ,J., FURTH,J.J., MALAMY,M. and ALEXANDER,M.: Proc.Natl. Acad.Sci.(Wash) 48, 1222 (1962)
(11) WEISS,S.B.: Proc.Natl.Acad.Sci.(Wash) 46, 1020 (1960)
(12) OGUR,M. and ROSEN,G.: Arch.Biochem. 25, 262 (1950)
(13) KIRBY,K.S.: Biochem.J. 64, 405 (1956)
(14) McLEAN,J.R., COHN,G.L., BRANDT,I.K. and SIMPSON,M.V.: J.Biol.Chem. 233, 657 (1958)
(15) WHEELER,G.P. and BENNETT,L.L.: Biochem.Pharmacol.11.353 (1962)
(16) NEUBERT,D.: Arch.exp.Path.u.Pharm.247, 372 (1964)
(17) unpublished experiments
(18) MEVES,H.: Arch.mikrosk.Anat. 72, 134 (1908)