Biochemical Pharmacology, Vol. 27, pp. 2139–2145.

0006-2952/78/0901-2139$02.00/0

EFFECTS OF RIFAMPICIN ON RNA AND PROTEIN SYNTHESIS IN ISOLATED RAT LIVER MITOCHONDRIA

WILLIAM C. BUSS
Department of Pharmacology, University of New Mexico School of Medicine, Albuquerque, NM, U.S.A.

ERNEST KUN
Department of Pharmacology and the Cardiovascular Research Institute, San Francisco Medical Center, San Francisco, CA, U.S.A.

(*Received* 3 *August* 1977; *accepted* 8 *November* 1977)

Abstract—Isolated rat liver mitochondria incorporated [^{3}H]UTP in an essentially linear fashion for at least 60 min in the presence and absence of exogenous ribonucleoside triphosphates. Preincubation of isolated mitochondria with rifampicin at 0° in the absence of ribonucleoside triphosphates produced a consistent inhibition of [^{3}H]UTP incorporation at 30° that ranged up to 75 per cent and was independent of preincubation time with rifampicin or the length of the assay period. Incorporation of [^{3}H]UMP into mitochondria prepared in the presence of the proteolytic enzyme Nagarse was similarly inhibited by rifampicin. When mitochondria prepared in the presence of Nagarse were preincubated with rifampicin, incorporation of [^{3}H]UTP was inhibited by approximately 75 per cent within 2 min, while [^{3}H]leucine incorporation continued in a linear fashion for 4–5 min before decreasing with first-order kinetics. These data demonstrate that rifampicin primarily affects RNA synthesis in mitochondria, and leads secondarily to an inhibition of protein synthesis. A 75 per cent inhibition of mitochondrial RNA synthesis resulting in a 45 per cent inhibition of mitochondrial protein synthesis permits an estimate of 3.3 mm as the half-life of mitochondrial RNA serving a messenger function. It is hypothesized that this rapidly turning-over mitochondrial RNA which is closely linked to protein synthesis serves a regulatory role in oxidative phosphorylation.

The rifamycin antibiotics were discovered in 1959 and have since become important chemotherapeutic agents [1, 2]. They are unusual compounds in that they block transcription by combining directly with the prokaryotic RNA polymerase rather than with the DNA template. They are highly specific in that this action is dependent upon binding to an apparently unique structural component of the prokaryotic RNA polymerase, the β-subunit. The binding of rifamycin antibiotic has been found to noncompetitively inhibit the binding of purine ribonucleoside triphosphates to RNA polymerase, suggesting a conformational interference with the site of nucleotide binding [3–5]. Interference with the nucleotide site by rifampicin appears to block initiation by preventing the transformation of the RNA polymerase–DNA template complex to the activated state.

In eukaryotic nuclei, multiple forms of RNA polymerase have been characterized [6–11]. Nuclear RNA polymerases from tissues ranging from plants to human placenta have been reported to be insensitive to rifamycin antibiotics in doses from 10 to 300 μg/ml [12–19]. Yeast mitochondrial RNA polymerase has been reported resistant to rifamycin antibiotics in concentrations up to 50 μg/ml in mitochondria that were highly swollen, as indicated by 90 per cent inhibition of RNA synthesis in the presence of actinomycin D. Mechanical disruption of mitochondria did not confer rifamycin antibiotic sensitivity, nor did preincubation of broken mitochondria with rifamycins prior to the addition of ribonucleoside triphosphates [16]. Mitochondrial transcription in intact or sonicated *Neurospora crassa* has been reported resistant to rifampicin in concentrations up to 40 μg/ml [20]. Purified *N. crassa* RNA polymerase was found to be inhibited by 6 μg/ml of rifampicin when poly d(AT) was used as a template, but endogenous RNA polymerase activity was not rifampicin sensitive [21].

Transcription in intact rat liver mitochondria has been reported to be sensitive to rifampicin at concentrations of 10–20 μg/ml. The synthesis of RNA in phosphate swollen mitochondria was found to be almost completely inhibited by 2 μg/ml of rifampicin [22]. RNA polymerase activity in intact rat liver mitochondria or in mitochondria prepared in the presence of the detergent Lubrol was also reported to be almost completely inhibited by 100 μg/ml of rifampicin, while RNA polymerase from Ehrlich ascites tumor cells was insensitive to rifampicin until high concentrations were attained [23]. In direct contrast, 50 μg/ml of rifampicin has been reported to be without effect in rat liver mitochondria swollen with phosphate or altered by digitonin [16]. Gadaleta *et al.* [24] reported that intact rat liver mitochondria were resistant to 100 μg/ml of rifampicin and ascribed this effect to mito-

chondrial membrane impermeability to rifampicin. When the mitochondria were swollen with phosphate, rifampicin was increasingly effective at 10–50 μg/ml. When the mitochondrial RNA polymerase was solubilized by sonication, enzyme activity was inhibited 50 per cent by 5 μg/ml, but required 50 μg/ml to inhibit 80 per cent of the enzyme activity. In partially purified rat liver mitochondrial RNA polymerase, inhibition ranged up to 85 per cent [25].

The data in this paper demonstrate that transcription in rat liver mitochondria is readily inhibited by rifampicin when the antibiotic is preincubated with mitochondria at 0°. Incorporation of [^{3}H]UMP into mitochondria prepared in the presence of the proteolytic enzyme Nagarse was similarly inhibited by rifampicin. Rifampicin inhibition was used to estimate the turn-over of the fraction of mitochondrial RNA directly related to protein synthesis and thus performing a messenger function.

MATERIALS AND METHODS

All materials listed were reagent or higher grades. $MgCl_2$, $MnCl_2$, $Na_2P_4O_7$, sucrose, D-mannitol, Trizma base, amino acids, crystalline bovine serum albumin (BSA) and malic and pyruvic acids were obtained from Sigma Chemical Co., St. Louis, MO, Trichloroacetic acid was purchased from J. T. Baker Chemical Co., Hayward, CA, scintanalyzed toluene from Fisher Scientific Co., Houston, TX, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) from CalBiochem, La Jolla, CA. Soluene, 2,5-diphenyloxazole (PPO), 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), Triton X-100 and other chemicals came from Packard Instrument Co., or Sigma Chemical Co., Downers Grove, IL, and Nagarse from Nagase Industry Co., Japan. ATP, ADP, CTP, GTP and UTP as the sodium salts, and crystalline uridine, were purchased from P-L Biochemicals, Milwaukee, WI. L[4,5-^{3}H]Leucine (58 Ci/m-mole), 5[^{3}H]uridine (27 Ci/m-mole) and 5[^{3}H]uridine-5′-triphosphate (1 Ci/m-mole as the sodium salt or 11.7 Ci/m-mole as the ammonium salt) were supplied by Amersham/Searle Corp., Arlington Heights, IL.

Preparation of mitochondrial fractions

Intact mitochondria. Male Sprague–Dawley rats (180–200 g) were obtained from Simonsen Laboratories, Gilroy, CA, and permitted to accommodate to the animal quarters for 3–4 days. All animals were then fasted 12 hr prior to sacrifice to reduce hepatic glycogen. Livers from six animals were used to prepare mitochondria according to the method of Schnaitman and Greenawalt [26] in a buffer containing 200 mM D-mannitol, 70 mM sucrose, 2 mM HEPES and 0.5 mg/ml of BSA adjusted to pH 7.4 at 0° with M Trizma base [sucrose–mannitol–HEPES (SMH) with BSA].

Liver homogenates were centrifuged at 560 *g* and the resultant supernatant fraction was centrifuged at 7000 *g* for 15 min. The crude mitochondrial pellet was gently homogenized by hand in one-half the original volume of SMH with BSA. The suspension was again centrifuged at 7000 *g* for 15 min, and the mitochondrial pellet was gently resuspended in one-fourth the original buffer volume. After recentrifugation at 7000 *g* for 15 min, the purified mitochondrial pellet was gently dispersed in SMH without BSA in the proportion of 1 ml SMH/liver. Mitochondrial protein was determined by a modified Biuret reaction [27], using crystalline BSA as standard. The concentration of protein in mitochondrial fractions prepared in the fashion described was generally close to 40 mg/ml (mean ± S.E.M. [20]: 43.82 ± 1.69 mg mitochondrial protein/ml).

Nagarse-treated mitochondria. For preparation of mitochondria in the presence of the bacterial proteolytic enzyme Nagarse, 1.6 to 1.8 mg Nagarse/g of liver was added to the primary liver homogenate. After mixing by inversion, the homogenate was permitted to stand for 2 min at 0° and a mitochondrial fraction was then prepared as previously described. The Nagarse was removed with decantation of the supernatant fraction from the crude mitochondrial pellet approximately 40 min later.

Assay conditions for [^{3}H]UTP and [^{3}H]L-leucine incorporations

Assays of RNA synthesis utilized 2.5 μCi [^{3}H]UTP (1 Ci/m-mole), and were performed in a total volume of 0.25 ml Tris–KCl buffer (Tris–HCl, 80 mM; KCl, 80 mM; pH 7.4 at 30°) or SMH (mitochondrial isolation medium adjusted to pH 7.4 at 30°). Experimental results were similar in both buffers. Those experiments in which exogenous ribonucleoside triphosphates were added contained 1.6 mM each of ATP, CTP, GTP and UTP, and 1.0 mM each of $MgCl_2$ and $MnCl_2$. This concentration of ribonucleoside triphosphates was in at least a 2-fold excess of that required to support maximal [^{3}H]UTP incorporation during the time periods examined. Rifampicin was added at concentrations of 10–100 μg/ml of incubation medium from a buffered stock solution of 400 μg/ml. Experiments that examined [^{3}H]L-leucine incorporation contained 2.5 μCi [^{3}H]L-leucine (58 Ci/m-mole) in 0.25 ml Tris–KCl or SMH buffer. Those experiments in which exogenous amino acids were added contained 5 mM malic acid, 5 mM pyruvic acid, 20 mM KH_2PO_4, 2 mM ADP, 5 mM $MgCl_2$, and up to 1.0 mg/ml of total amino acids in molar proportions found in the cell as defined by Roodyn *et al.* [28].

Reaction ingredients were combined in 10 × 75 pyrex tubes (3.0 ml) at 0°. Mitochondria at a concentration of 1.2 to 1.5 mg of mitochondrial protein/assay were added to individual tubes 30 sec apart to initiate assays. Assay tubes were immediately transferred to a Dubnoff shaking water bath and incubated at 30° for various time periods. Preincubation at 0° was done in an ice-water slurry. Incubations were terminated by the addition of 0.25 ml of ice-cold SMH or Tris–KCl containing 100 mM $Na_2P_4O_7$ or 2 mM L-leucine to reduce non-specific label binding. Immediately afterward, 1.0 ml of 10% trichloroacetic acid (TCA) containing $Na_2P_4O_7$ or L-leucine was added to precipitate macromolecular material. The samples were vortexed and 5% TCA containing $Na_2P_4O_7$ or L-leucine was added to make a final volume of approximately 2.5 ml. Tubes were held on ice for 10 min, then centrifuged for 10 min at 17,300 *g*. The supernatant fraction was carefully

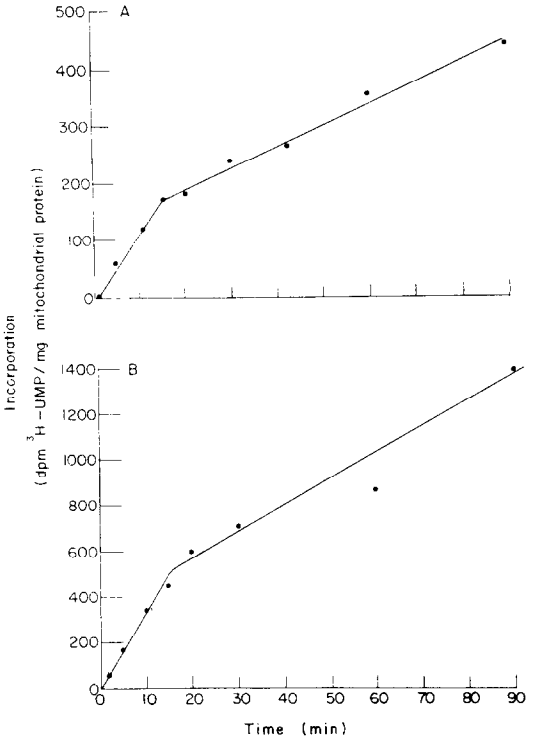

Fig. 1. Representative incorporation values *in vitro* for [^{3}H]UTP at 30° in the presence (A) and absence (B) of exogenous ribonucleoside triphosphates, Mg^{2+} and Mn^{2+}. Mitochondria (1.04 and 1.22 mg protein/assay in A and B respectively) were incubated for the times shown at 30° in 0.25 ml Tris–KCl buffer (pH 7.4), with 2.5 μCi [^{3}H]UTP. Incubations were performed in the presence (A) and absence (B) of ribonucleoside triphosphates (1.6 mM each of ATP, CTP, GTP and UTP), Mg^{2+} and Mn^{2+} (1.0 mM each). The reactions were quenched and the acid-insoluble material was prepared for counting as described in Materials and Methods. Each point is the average for two values obtained using mitochondria isolated from six pooled livers. Incorporation values at 90 min represented 30.5 and 0.616 pmoles UMP/mg of mitochondrial protein in A and B respectively. Counting efficiencies: (A) 51 per cent, and (B) 47 per cent.

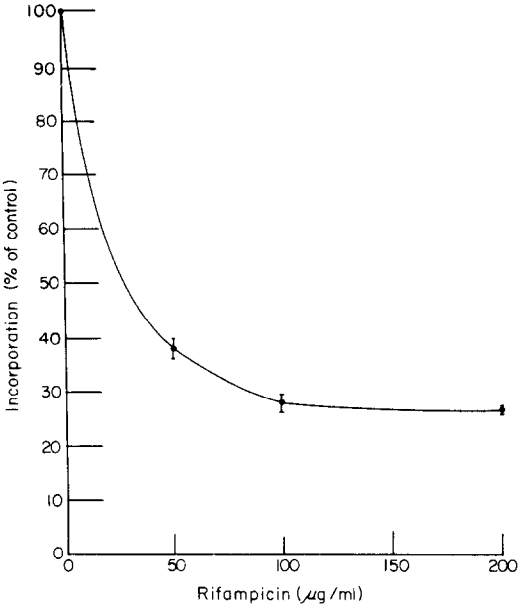

Fig. 2. Inhibition of [^{3}H]UTP incorporation *in vitro* at 30° into mitochondria after preincubation for 30 min at 0°. Assays were performed in 0.25 ml Tris–KCl buffer (pH 7.4) for 30 min at 30° after preincubation for 30 min at 0° in the presence of rifampicin. Each point shown is the mean ± S.E.M. for four values from two separate experiments, each using the mitochondrial fraction isolated from six pooled livers. Experiment 1 contained 1.16 mg of mitochondrial protein and 2.4 μCi [^{3}H]UTP, while Expt. 2 contained 1.24 mg of mitochondrial protein and 2.5 μCi [^{3}H]UTP. An incorporation value of 100 per cent represents 1.37 and 0.79 pmoles UMP/mg of mitochondrial protein, or 3109 and 1792 dis./min, in Expts. 1 and 2 respectively. Counting efficiencies: (1) 46 per cent, and (2) 36 per cent.

aspirated off, and the pellet dissolved with vortexing in 1.0 ml of 0.1 M NaOH. Macromolecular material was then reprecipitated with 1.0 ml of 10% TCA, followed by the addition of 0.5 ml of 5% TCA. Samples were placed in ice for 10 min and then recentrifuged at 17,300 *g*. The pellet was dissolved, precipitated and centrifuged a second time. The tubes were then inverted to permit the washed pellets to drain at 2°. Pellets were dissolved in 0.5 ml of tissue solubilizer (e.g. Soluene) and taken up in a 15-ml toluene scintillation mixture (4.0 g PPO and 0.1 g POPOP/liter of toluene) for counting in a Packard 3320 TriCarb scintillation spectrometer.

At mitochondrial protein concentrations of 1.2 to 1.5 mg/assay, a slight yellow color appeared upon dissolving the mitochondrial pellet in solubilizer. A quenching curve for mitochondrial protein was prepared by dissolving up to 5 mg of mitochondrial protein in solubilizer and toluene scintillation mixture, and then adding known amounts of tritium as [^{3}H]UTP. A similar quenching curve was obtained to correct counts made in the presence of rifampicin, which remained bound to mitochondrial proteins through the pellet washing and imparted color to the counting solution. Counting efficiency was generally very close to 40 per cent (range 36–51 per cent).

RESULTS

Effects of ribonucleoside triphosphates, Mg^{2+} *and* Mn^{2+} *on mitochondrial* [^{3}H]*UTP incorporation*

The incorporation of [^{3}H]UTP or [^{3}H]uridine into isolated mitochondria incubated at 30° in a Tris–KCl or sucrose–mannitol–HEPES (SMH) buffer at pH 7.4 was essentially linear for at least 60 min. Incorporation was independent of the addition of exogenous ribonucleoside triphosphates, Mg^{2+} or Mn^{2+}. However, when exogenous ribonucleoside triphosphates were added in concentrations in excess of those required for maximal incorporation, [^{3}H]UTP incorporation calculated on the basis of label dilution was generally at least an order of magnitude larger then incorporation observed utilizing endogenous substrate pools. This is demonstrated in Fig. 1, which gives representative values for [^{3}H]UTP incorporation into mitochondria in the presence and absence of exogenous ribonucleoside

Table 1. Effects of preincubating mitochondria with rifampicin at 0° for various times on [^{3}H]UTP incorporation at 30° in the absence of exogenous ribonucleoside triphosphates*

Preincubation time at 0° (min)	Incorporation (pmoles UMP/mg protein) Rifampicin (μg/ml) 0	100	Inhibition (%)
10	0.584	0.145	75.2
30	0.586	0.162	72.3
60	0.556	0.169	69.6

* Mitochondria (1.2 mg protein/assay) were preincubated for the times indicated at 0° in 0.25 ml of Tris–KCl buffer (pH 7.4) containing 0 or 100 μg/ml of rifampicin, followed by the addition of 2.5 μCi[^{3}H]UTP for a 30-min assay at 30°. Figures are the average of two values obtained from mitochondria isolated from six pooled livers. An incorporation value of 0.50 pmole UMP represented 492 dis./min. Counting efficiency: 43 per cent.

Table 2. Effects of preincubating mitochondria with rifampicin for 30 min at 0° on [^{3}H]UTP incorporation at 30° for various times in the absence of exogenous ribonucleoside triphosphates*

Incubation time at 30° (min)	Incorporation (pmoles UMP/mg protein) Rifampicin (μg/ml) 0	100	Inhibition (%)
15	0.246	0.056	77.2
90	1.200	0.250	77.4

* Mitochondria (1.2 mg protein/assay) were preincubated for 30 min at 0° in 0.25 ml of Tris–KCl buffer (pH 7.4) with 0 or 100 μg/ml of rifampicin, followed by the addition of 2.5 μCi [^{3}H]UTP for assay for the times indicated at 30°. Figures are the average of two values obtained using mitochondria isolated from six pooled livers. An incorporation value of 0.25 pmole UMP represented 205 dis./min. Counting efficiency: 36 per cent.

triphosphates, Mg^{2+} and Mn^{2+}. With excess exogenous ribonucleoside triphosphates, the results were identical in the presence or absence of added Mg^{2+} and Mn^{2+}. Acid-insoluble incorporation was 90–95 per cent solubilized by incubation with 5% TCA for 10 min at 100°, and 90 per cent solubilized by heating with 0.5 M NaOH for 1 hr at 37°. These data suggest that the label was incorporated almost exclusively into an RNA fraction.

Rifampicin inhibition of [^{3}H]UTP incorporation into mitochondria in vitro

When intact mitochondria were preincubated with rifampicin at 0° in the absence of exogenous ribonucleoside triphosphates, the incorporation of [^{3}H]UTP was consistently and dramatically decreased, as shown in Fig. 2. Experiments were then performed to determine whether the time of preincubation at 0° was a factor in rifampicin inhibition. Table 1 demonstrates that preincubation with rifampicin at 0° for periods of 10, 30 or 60 min did not appreciably alter the degree of [^{3}H]UTP incorporation or rifampicin inhibition. When mitochondria were preincubated with rifampicin at 0° for 30 min, and then incubated with [^{3}H]UTP at 30° in the continued presence of rifampicin for 15 or 90 min (Table 2), the degree of rifampicin inhibition remained the same. The ratio of [^{3}H]UTP incorporation values for rifampicin-treated/control mitochondria was 0.22 for both 15- and 90-min incubations. Rifampicin effects were not observed in the presence of exogenous ribonucleoside triphosphates.

Rifampicin inhibition of [^{3}H]UTP incorporation into mitochondria prepared with the proteolytic enzyme Nargarse

In an attempt to determine whether the mitochondrial membrane was a limiting factor in rifampicin effects, mitochondrial fractions were treated with the proteolytic enzyme Nagarse to alter or remove the outer mitochondrial membrane. When

Table 3. Effects of pretreating mitochondria with the proteolytic enzyme Nagarse on [^{3}H]UTP incorporation at 30° in the presence and absence of rifampicin*

Ribonucleoside triphosphates (1.6 mM)	Rifampicin (100 μg/ml)	Preincubation at 0° (30 min)	Incorporation (pmoles UMP/mg protein)	Inhibition (%)
−	−	+	1.13	
−	+	+	0.21	81.2
−	−	−	1.10	
−	+	−	0.21	81.2
+	−	+	30.0	
+	+	+	10.3	65.7

* Nagarse-treated mitochondria (1.2 mg protein/assay), with or without preincubation for 30 min at 0°, were assayed for 30 min at 30° in 0.25 ml of SMH buffer (pH 7.4) containing 2.5 μCi [^{3}H]UTP. Rifampicin (100 μg/ml) and ribonucleoside triphosphates (1.6 mM each of ATP, CTP, GTP and UTP) were added as indicated. Figures are the average of two values obtained from mitochondria isolated in the presence of Nagarse from six pooled livers, as described in Materials and Methods. Incorporation values of 1.0 pmole UMP in the absence, and 30 pmoles UMP in the presence, of ribonucleoside triphosphates represented 2275 and 840 dis./min respectively. Counting efficiency: 40 per cent.

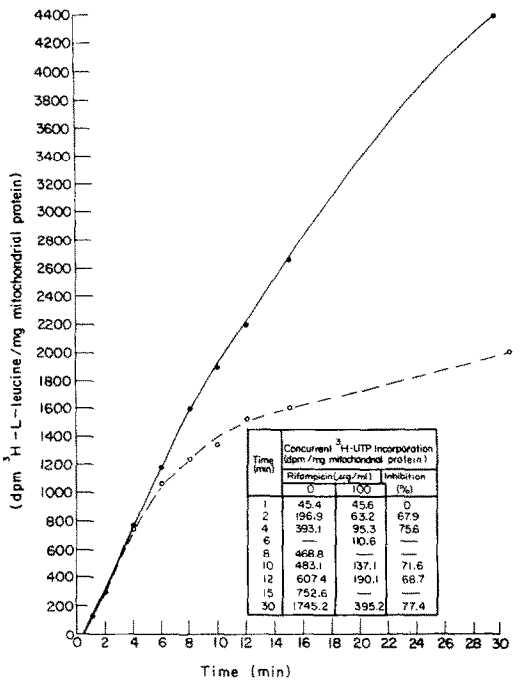

Fig. 3. Incorporation *in vitro* of [³H]L-leucine and [³H]UTP into Nagarse-treated mitochondria at 30° in the presence and absence of rifampicin after preincubation for 30 min at 0°. Nagarse-treated mitochondria, prepared as described in Materials and Methods, were preincubated with, and without, rifampicin (100 μg/ml) for 30 min at 0° in flasks containing 5.0 ml of SMH buffer. [³H]L-leucine was added to two flasks and [³H]UTP was added to two additional flasks. All four flasks were then incubated at 30°. Aliquots of 0.5 ml, containing 0.69 mg of mitochondrial protein and 2.5 μCi[³H]UTP or [³H]L-leucine, were removed at the times indicated. Cold L-leucine was added to the two flasks receiving [³H]UTP in concentrations equivalent to the amount of [³H]L-leucine added to the remaining two flasks. Ethanol and cold UTP were added to the two flasks receiving [³H]L-leucine in concentrations equivalent to the amount of each added as [³H]UTP in 50% ethanol to the other two flasks. Thus, all flasks contained identical substrates, differing only in regard to the labeled material and the presence and absence of rifampicin. In the graph, (●—●) represents control [³H]L-leucine incorporation, and (○---○) represents [³H]L-leucine incorporation in the presence of 100 μg/ml of rifampicin. An incorporation value of 1000 dis./min of [³H]L-leucine represented 0.022 pmole L-leucine/mg of mitochondrial protein. The mean ± S.E.M. for the inhibition of incorporation of [³H]UTP in the presence of rifampicin over the 30-min assay period (inset table) was 72.24 ± 1.67 per cent. An incorporation value of 1745 dis./min for [³H]UMP (30 min, 0 rifampicin) represented 0.77 pmole UMP/mg of mitochondrial protein. Counting efficiency: 39 per cent.

mitochondria which had been prepared in the presence of Nagarse (see Materials and Methods) were examined for rifampicin effects on [³H]UTP incorporation, dramatic rifampicin effects were found when the Nagarse-treated mitochondria were preincubated at 0° or received no preincubation, and in the presence or absence of exogenous ribonucleoside triphosphates (Table 3). Similar results (data not shown) were obtained in mitoplasts prepared with the glucosidic detergent digitonin according to the method of Schnaitman and Greenawalt [26].

Relationship between the inhibition of [³H]*UTP and* [³H]*leucine incorporation in mitochondria prepared in the presence of Nagarse*

In order to determine the simultaneous effects of rifampicin on [³H]UTP and [³H]leucine incorporation, and the temporal relationship between these incorporations, mitochondria prepared in the presence of Nagarse were preincubated at 0° in the presence and absence of rifampicin before assay at 30°, utilizing [³H]L-leucine or [³H]UTP. Exogenous ribonucleoside triphosphates or amino acids were not used. The results of a representative experiment are shown in Fig. 3.

In Fig. 3, rifampicin inhibition of [³H]UTP was not observed at 1 min, but at 2 min it was 94.0 per cent of the mean value of rifampicin inhibition occurring over the subsequent 28-min period of the 30-min assay. After 2 min, rifampicin inhibition of [³H]UTP incorporation remained constant over the incubation period with a mean ± S.E.M. of 72.24 ± 1.67 per cent. In contrast, inhibition of [³H]leucine incorporation began at approximately 5 min, and decreased with first-order kinetics from approximately 6 to 12 min, reaching a new state of relatively linear incorporation beyond the period of 12 min which extended over the remaining 18 min of the incubation period. [³H]leucine incorporation was inhibited by 45 per cent at the end of the 30-min assay period.

DISCUSSION

Isolated mitochondria incorporated [³H]UTP in an essentially linear fashion for up to 90 min. The incorporation of [³H]UTP was not dependent upon exogenous ribonucleoside triphosphates or divalent metallic cations, but was stimulated in the presence of nucleotides. This is demonstrated in Fig. 1, in which incorporation in the absence of exogenous nucleotides is undoubtedly due to endogenous mitochondrial substrate pools [29, 30]. The change in the incorporation rate that occurs at 12–15 min (Fig. 1) was observed both in the presence and absence of exogenous ribonucleoside triphosphates. A similar phenomenon has been observed for [¹⁴C]ATP incorporation [31]. This change in kinetics is thought to be due to nuclease activity, which has been shown to interrupt the linearity of incorporation [32]. Incorporation was primarily polymerization into macromolecular RNA, as shown by its sensitivity to alkali treatment.

When mitochondria were preincubated with rifampicin at 0° in the absence of exogenous ribonucleoside triphosphates, consistent dose-dependent decreases in [³H]UTP incorporation were observed. Rifampicin concentrations up to 200 μg/ml did not result in an inhibition greater than 75 per cent. Table 1 demonstrates that the degree of inhibition of [³H]UTP incorporation observed upon preincubation of mitochondria with rifampicin at 0° was independent of the preincubation time, and remained close to 75 per cent. Table 2 demonstrates that the inhibition remained very close to 75 per cent regardless of the length of the assay period at 30°. This inhibition could not be observed when intact mitochondria were preincubated with rifampicin at

0° in the presence of exogenous ribonucleoside triphosphates.

Attempts were next made to determine whether the sensitivity of mitochondrial [^{3}H]UTP incorporation to rifampicin was limited by mitochondrial membrane permeability to the antibiotic. When mitochondria were prepared with the proteolytic enzyme Nagarse, incorporation was inhibited by rifampicin to the same degree in the absence of ribonucleoside triphosphates whether the mitochondria were preincubated with rifampicin at 0° or when this step was omitted and rifampicin was added with [^{3}H]UTP at 30° for the incorporation assay (Table 3). The presence of exogenous ribonucleoside triphosphates markedly increased incorporation, but rifampicin inhibition of [^{3}H]UMP incorporation was only slightly reduced from that value seen in the absence of exogenous triphosphates. The degree of rifampicin inhibition observed was similar in intact mitochondria preincubated at 0° with rifampicin and in mitochondria prepared with Nagarse and incubated at 30° with rifampicin.

In a final series of experiments, we examined the relationship between mitochondrial RNA and protein synthesis using rifampicin. Replicate flasks were used to examine [^{3}H]UTP and [^{3}H]L-leucine incorporation after preincubation of Nagarse-treated mitochondria with rifampicin at 0° for 30 min in the absence of exogenous ribonucleoside triphosphates and amino acids (Fig. 3). [^{3}H]UTP incorporation was essentially maximally inhibited (72.24 per cent) within 2 min, while [^{3}H]L-leucine incorporation in the presence of rifampicin continued for 4 min before significant deviations from the control incorporation curve were observed. The fact that the curves in Fig. 3 do not pass through the origin represents the time required for the flasks to warm from 0 to 30°. Figure 3 provides direct evidence that rifampicin inhibits mitochondrial transcription and secondarily results in decreases in mitochondrial protein synthesis. We propose that the change in the kinetic rate for mitochondrial [^{3}H]L-leucine incorporation represents the decay of RNA in mitochondria that is linked to mitochondrial protein synthesis, i.e. serves a messenger function. When the data from Fig. 3 were plotted as log incorporation rate vs time, it demonstrated that the synthesis of mitochondrial protein as determined by [^{3}H]L-leucine incorporation decayed with first-order kinetics from approximately 6 to 12 min, with a half-life of 3.3 min. Thus, approximately 50 per cent of rat liver mitochondrial RNA turns over under these experimental conditions at a rate that is close to bacterial RNA turn-over (e.g. 1.9 ± 0.5 min in *Escherichia coli* [33]).

Rat liver mitochondrial products synthesized *in vitro* have been found to be similar in number and size distribution to those synthesized *in vivo* [34–36]. Moreover, RNA synthesized by isolated rat liver mitochondria demonstrates sedimentation properties of mRNA [34]. Studies *in vivo* have suggested that it requires up to 24 hr to appreciably label mitochondrial rRNA [37, 38]. It has also been suggested that the mitochondrial RNA labeled *in vitro* within 1 hr is either mRNA or rRNA precursor [39]. Our demonstration of the temporal relationship of rifampicin-sensitive RNA synthesis to ongoing mitochondrial protein synthesis suggests that the transcription product contains mRNA. Therefore, the synthesis of an appreciable amount of the mitochondrial RNA which serves a messenger function is inhibited by rifampicin within minutes.

Gamble and McCluer [40] have demonstrated that isolated bovine heart mitochondria synthesize both stable and unstable RNA, and that rifampicin inhibits the synthesis of both types. The authors used rifampicin effects on mitochondrial [^{3}H]uridine and [^{14}C]leucine incorporation to determine that RNA in isolated mitochondria has a half-life of 1.4 min. Grant and Poulter [41] used rifampicin to determine that isolated mitochondria from the myxomycete *Physarum polycephalum* produce an RNA with a messenger function that turns over with a half-life of 2–3 min. In yeast, when acriflavin was used to prevent transcription of the yeast mitochondrial RNA, cycloheximide resistant protein synthesis decreased with first-order kinetics with a half-life of 3–4 min [42]. A similar half-life for mitochondrial protein synthesis has been observed in HeLa cells [43]. Gadaleta *et al.* [44] reported an average mitochondrial RNA half-life of 5.8 and 4.3 min for thyroidectomized rats treated with buffer and triiodothyronine respectively.

The mitochondrial respiratory chain is controlled by both mitochondrial and nuclear genomes. The bulk of mitochondrial proteins (85–90 per cent) is believed to be synthesized on cytoplasmic ribosomes [45]. Mitochondrial RNA serves as a template for the transcription of mitochondrial ribosomal and transfer RNA, and messenger RNA for up to approximately twenty proteins [42, 46]. Mitochondrial products identified in the presence of cycloheximide or synthesized *in vitro* in mitochondria from yeast and rat liver cells have ranged in number from five to twenty and in molecular mass from 7,800 to 50,000 daltons [36, 42, 47, 48]. The identified products of mitochondrial transcription include subunits of cytochromes *b*, *c* and c_1, cytochrome *c* oxidase, and ATPase [35, 36, 45, 49, 50]. Mutations which significantly alter, or result in a loss of, mitochondrial DNA (e.g. yeast petite mutants) result in the loss of both the respiratory and phosphorylating capacities of mitochondria [36]. Petite mutants contain abnormal inner mitochondrial membranes, abnormal Krebs cycle enzymes, and an incomplete respiratory chain [51]. Ethidium bromide, a dye that intercalates between bases in mitochondrial DNA [52], has been found to inhibit oxidative phosphorylation [53]. Mitochondrial products appear in many instances to be single subunits of multi-subunit enzymes that are complemented by nuclear products, and these mitochondrial products may thus serve a unique structural and functional role by permitting the assembly of inner mitochondrial membranes [45, 54]. Cytoplasmically produced proteins have been shown to stimulate mitochondrial DNA and protein synthesis [50, 55].

We hypothesize that the rapidly turning-over mRNA of the mitochondrial genome that we observed to be blocked by rifampicin *in vitro* encodes for proteins with a direct role in oxidative phosphorylation. The high turn-over rate of this mito-

chondrial transcription product makes it a potential site for the cytoplasmic regulation of mitochondrial ATP production. The observation that the product of bovine heart mitochondrial transcription turns over with a half-life of 1.4 min, in contrast to estimates (including our own) of 3–4 min in rat liver, a tissue with less demanding energy requirements, supports this hypothesis. This hypothesis would predict differential turn-over rates for mitochondrial transcription product(s) depending upon the energy demands of the tissue. We are currently examining the relationship of mitochondrial transcription to oxidative phosphorylation in order to test this hypothesis further.

Acknowledgements—This work was funded through an NIH Training Grant to the Department of Pharmacology, University of California, San Francisco, and performed when W. C. Buss was a post-doctoral appointee.

REFERENCES

1. S. Furesz, *Antibiot. Chemother.* **16,** 316 (1970).
2. W. Wehrli and M. Staehelin, *Bact. Rev.* **35,** 290 (1971).
3. C. W. Wu and D. A. Goldthwait, *Biochemistry* **8,** 4450 (1969).
4. C. W. Wu and D. A. Goldthwait, *Biochemistry* **8,** 4458 (1969).
5. D. A. Goldthwait, D. A. Anthony and C. W. Wu, in *Proc. First Lepetit Colloq. on RNA Polymerase and Transcription* (Ed. L. Silvestri), p. 10. North Holland, Amsterdam (1970).
6. J. R. Tata, in *Progress in Nucleic Acid Research and Molecular Biology* (Eds J. N. Davidson and W. E. Cohen), p. 191. Academic Press, NY (1966).
7. R. G. Roeder and W. J. Rutter, *Nature, Lond.* **224,** 234 (1969).
8. R. C. Roeder and W. J. Rutter, *Proc. natn. Acad. Sci. U.S.A.* **65,** 675 (1970).
9. C. J. Chesterton and P. H. W. Butterworth, *Fedn Eur. Biochem. Soc. Lett.* **15,** 181 (1971).
10. C. Kedinger, P. Nuret and P. Chambon, *Fedn Eur. Biochem. Soc. Lett.* **15,** 169 (1971).
11. D. R. McNaughton, G. R. Klassen and H. B. LeJohn, *Biochem. biophys. Res. Commun.* **55,** 468 (1975).
12. W. Wehrli, J. Nuesch, F. Knusel and M. Staehelin, *Biochim. biophys. Acta* **157,** 215 (1968).
13. S. T. Jacob, E. M. Sajdel and H. N. Munro, *Biochem. biophys. Res. Commun.* **32,** 831 (1968).
14. E. Nagl, *Naturwissenschaften* **57,** 458 (1970).
15. S. Dezellee, A. Sentenac and P. Fromageot, *Fedn Eur. Biochem. Soc. Lett.* **7,** 220 (1970).
16. E. Wintersberger and U. Wintersberger, *Fedn Eur. Biochem. Soc. Lett.* **6,** 58 (1970).
17. J. E. Byfield, Y. C. Lee and L. R. Bennett, *Biochim. biophys. Acta* **204,** 610 (1970).
18. J. J. Furth, A. Nicholson and G. E. Austin, *Biochim. biophys. Acta* **213,** 124 (1970).
19. H-P. Voigt, R. Kaufmann and H. Matthaei, *Fedn Eur. Biochem. Soc. Lett.* **10,** 257 (1970).
20. F. Herzfeld, *Hoppe-Seyler's Z. physiol. Chem.* **351,** 658 (1970).
21. H. Kuntzel and K. P. Schafer, *Nature, New Biol.* **231,** 265 (1971).
22. Zh. G. Schmerling, *Biochem. biophys. Res. Commun.* **37,** 965 (1969).
23. R. Jackisch, A. Jung, W. Schlegel and D. Mayer, *Hoppe-Seyler's Z. physiol. Chem.* **353,** 1705 (1972).
24. M. N. Gadaleta, M. Greco and C. Saccone, *Fedn Eur. Biochem. Soc. Lett.* **10,** 54 (1970).
25. B.D. Reid and P. Parsons, *Proc. natn. Acad. Sci. U.S.A.* **68,** 2830 (1971).
26. C. Schnaitman and J. W. Greenawalt, *J. Cell Biol.* **38,** 158 (1968).
27. A. G. Gronall, C. S. Barderwill and M. M. David, *J. biol. Chem.* **177,** 751 (1949).
28. D.B. Roodyn, P. J. Reis and T. S. Work, *Biochem. J.* **80,** 9 (1961).
29. H. W. Heldt and M. Klingenberg, *Biochem. Z.* **343,** 433 (1965).
30. D.Neubert and H. Helge, *Biochem. biophys. Res. Commun.* **18,** 600 (1965).
31. C. Saccone, M. N. Gadaleta and E. Quagliariello, *Biochim. biophys. Acta* **138,** 174 (1967).
32. T. C. Spelsberg and J. T. Wilson, *Biochem. J.* **154,** 439 (1976).
33. A. Puga M-T. Borras, E. S. Tessman and I. Tessman, *Proc. natn. Acad. Sci. U.S.A.* **70,** 2171 (1973).
34. C. Saccone, M. N. Gadaleta and R. Gallerani, *Eur. J. Biochem.* **10,** 61 (1969).
35. J. L. Coote and T. S. Work, *Eur. J. Biochem.* **23,** 564 (1971).
36. A. Tzagoloff, M. S. Rubin and M. F. Sierra, *Biochim. biophys. Acta* **301,** 71 (1973).
37. E. Zylber, C. Vesco and S. Penman, *J. molec. Biol.* **44,** 195 (1969).
38. B. Bartoov, R. S. Mitra and K. B. Freeman, *Biochem. J.* **120,** 455 (1970).
39. S. Fukamachi, B. Bartoov and K. B. Freeman, *Biochem. J.* **128,** 299 (1972).
40. J. G. Gamble and R.H. McCluer, *J. molec. Biol.* **53,** 557 (1970).
41. W. D. Grant and R. T. M. Poulter, *J. molec. Biol.* **73,** 439 (1973).
42. L. W. Wheeldon, *Biochim.* **55,** 805 (1973).
43. M. Lederman and G. Attardi, *Biochem. biophys. Res. Commun.* **40,** 1492 (1972).
44. M. N. Gadaleta, N. DiReda, G. Bove and C. Saccone, *Eur. J. Biochem.* **51,** 495 (1975).
45. N. F. Gonzalez-Cadavid, *Subcell. Biochem.* **3,** 275 (1974).
46. G. Schlanger and R. Sager, *Proc. natn. Acad. Sci. U.S.A.* **71,** 1715 (1974).
47. R. L. Melnick, H. M. Rinberg, J. Maguire and L. Packer, *Biochim. biophys. Acta* **311,** 230 (1973).
48. S. Kuzela, U. Krempasky, J. Kolarov and V. Ujhazy, *Eur. J. Biochem.* **58,** 483 (1975).
49. G. Jacki and W. Sebald, *Eur. J. Biochem.* **54,** 97 (1975).
50. N. G. Ibrahim and D. S. Beattie, *J. biol. Chem.* **251,** 108 (1976).
51. P. Borst, *A. Rev. Biochem.* **41,** 333 (1972).
52. W. Klietmann, N. Sato and M. M. K. Nass, *J. Cell Biol.* **58,** 11 (1973).
53. E. S. Higgins, B. L. Dunlavey, W. H. Friend and K. S. Rogers, *Proc. Soc. exp. Biol. Med.* **149,** 1055 (1975).
54. H. D. Hoberman, *Cancer Res.* **35,** 3332 (1975).
55. M. A. D'Agostino, K. M. Lowry and G. F. Kalf, *Archs Biochem. Biophys.* **166,** 400 (1975).