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# Effect of diphtheria toxin fragment A on energy coupling in mitochondria. Studies on mouse liver mitoplasts

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**Abstract.** The effect of intact diphtheria toxin and of its fragment A on protein synthesis in mouse liver mitoplasts (digitonin-treated mitochondria) was studied. Fragment A inhibited protein synthesis in intact mitoplasts to the same extent as the uncoupler, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, but similar effects were not observed in lyzed mitoplasts. Intact diphtheria toxin was without effect in either case.

Fragment A strongly stimulated mitochondrial ATPase activity. At concentrations which efficiently inhibited mitochondrial protein synthesis and stimulated ATPase activity, fragment A had no effect on the intramitochondrial concentration of nicotinamide adenine dinucleotides. Moreover, it did not catalyze ADP ribosylation of mitochondrial proteins. The results indicate that the effects observed did not involve the $NAD^+$-glycohydrolase activity of fragment A.

[$^{125}$I]-Labelled fragment A was bound to mitoplasts to about the same extent as the labelled intact diphtheria toxin.

The present results suggest that fragment A of diphtheria toxin is capable of inhibiting the energy coupling in mitoplasts, thereby inhibiting protein synthesis. The detailed mechanism of the uncoupling and its possible physiological significance remains to be elucidated

**Keywords.** Diphtheria toxin; fragment A; mitochondria; mitoplasts; uncoupling.

## Introduction

Recently we have been involved in a study of protein synthesis in mouse liver mitochondria in an attempt to characterize the polypeptides formed. For this purpose mitoplasts, *i.e.* mitochondria devoid of its outer membrane, were used. In order to ascertain that the polypeptides formed were genuine mitochondrial products, the effect ofvarious inhibitors, such as chloramphenicol and cycloheximide, was tested (Ashwell and Work 1970; Schatz and Mason, 1974). The sensitivity to diphtheria toxin was used as an additional criterion since this toxin is known to be a potent inhibitor of the cytoplasmic elongation factor 2 (Gill *et al.*, 1969), but not of the corresponding factor from yeast mitochondria (Richter and Lipmann, 1970). In agreement with the above finding, protein synthesis in mammalian mitoplasts was not inhibited by intact diphtheria toxin. Since the amount of fragment A which carries the toxic activity, varies in different toxin preparations, we tested the effect

Abbreviations used: DTT, Dithiothreitol.

of purified fragment A in this system. Unexpectedly, fragment A, in contrast to intact diphtheria toxin, inhibited protein synthesis in mitoplasts. Here we report these results as well as studied designed to elucidate the nature of the effect. The data presented indicate that the inhibitory effect of fragment A on protein synthesis in mitoplasts is indirect, and due to a previously unkown property of fragment A, *viz.* the ability to inhibit energy coupling.

## Materials and methods

### *Materials*

Diphtheria toxin was obtained from Connaught Laboratories Ltd., Ontario, Canada, digitonin from Sigma Chemical Co., St. Louis, Missouri, USA and [$^{35}$S]-methionine and [$^{14}$C]-NAD$^{+}$ from The Radiochemical Centre, Amersham, England.

### *Preparation of mitoplasts*

All solutions used for the preparation of mitoplasts were sterile filtered and all centrifugations were carried out at 2°C in a Sorvall HB-4 rotor, using sterile 30 ml Corex tubes. Liver mitochondria were isolated from 6 weeks old Swiss mice, (Romslo and Flatmark, 1973). The final mitochondrial pellet was suspended in Hepes sucrose medium at a final concentration of 7.5 mg protein/ml, and treated with a 3 mg/ml solution of digitonin in the same buffer to a final concentration of 0.2 mg digitonin/mg protein (Chan *et al.*, 1970). The digitonin-treated mitochondria were slowly stirred at 2° C for 15 min and diluted with Hepes sucrose medium to a final concentration of 5 mg protein/ml. The mitoplasts were pelleted by centrifugation at 10,500 *g* for 10 min. The pellet was resuspended in 20 ml of Hepes sucrose medium and centrifuged at 500 g for 5 min. Mitoplasts in the supernatant were pelleted by centrifugation at 7,000 g for 10 min and suspended in a buffer containing 5 mM Hepes (pH 7.4) and 150 mM KCl at a final protein concentration of 60 mg/ml. The mitoplasts were used immediately after preparation.

### *Isolation of the mitochondrial matrix and inner membrane fraction*

Mitoplasts were resuspended in Hepes sucrose and sonicated in a pyrex tube (5 ml) cooled in ice-water. The sonifier (Branson, model S-75) was operated at a current output of 4A, and the suspension was sonicated five times for 30 s with 1 min intervals in order to keep the temperature below 10° C. The sonicated mitoplasts were centrifuged at 165,000 *g* for 2 h, using the 50 Ti rotor (fixed angle, $R_{min}$= 3.8 cm and $R_{max}$= 8.1 cm) of the Spinco L2-65 B ultracentrifuge, operated at 4°C. The matrix fraction (supernatant) and the inner membrane fraction (pellet) were collcted.

### *Preparation of diphtheria toxin fragment A*

Fragment A of the toxin was prepared by trypsin treatment of intact diphtheria toxin, followed by dissociation with dithiothreitol (DTT), according to Drazin *et al.* (1971). The A-fragment was dialyzed against 10 mM Hepes buffer (pH 7.4) contaning 1 mM DTT.

*Assay of protein synthesis in mitoplasts*

Incubations of mitoplasts for measurement of protein synthesizing activity were carried out in open tubes with free access to oxygen. Incorporation of [$^{35}$S]-methionine into proteins insoluble in hot trichloroacetic acid, was assayed under maximally energized conditions, *i.e.* respiration was supported by NADH-linked substrates and ATP plus ADP were present in the incubation medium. For experimental details, see legend to figure 1.

*Assay of ATPase activity*

Mitoplasts were incubated at 37°C under conditions used for protein synthesis, with the following modifications: Amino acids were omitted from the reaction mixture which contained 3 mM MgATP. The reaction was stopped after various time intervals by withdrawing 30 µl aliquots of the mixture into 200 µl 5% (v/v) cold perchloric acid. The protein precipitate was removed by centrifugation. The supernatant was diluted (three times), with 0.45 M potassium phosphate, pH 3.93, and 20 µl aliquots of the supernatants were used for analysis of adenine nucleotides.

The adenine nucleotides were assayed by high-performance liquid chromatography (HPLC) as described (Normann and Flatmark, 1978; Terland *et al.*,1979). The chromatographic separation of the nucleotides was achieved at 20°C on a strong anion exchanger (Partisil 10-SAX), prepacked from Whatman in a 25 cm stainless steel column of 4.6 mm inner diameter. The details are given in legend to figure 3.

*Assay of ADP-ribosylating activity*

The ability of fragment A of diphtheria toxin to ADP-ribosylate mitochondrial and cytosolic proteins was assayed by measuring the incorporation of radioactivity from [$^{14}$C]$NAD^+$, labelled in the adenosine moiety, into trichloroacetic acid-insoluble material, according to the procedure of Gill and Dinius (1973).

*Assay of toxin binding*

Two mg of diphtheria toxin was iodinated, using lactoperoxidase as described earlier (Sandvig *et al.*, 1976). The iodinated protein was purified by molecular sieve chromatography (twice) in 50 mM Tris-HCl buffer. The first chromatography was carried out in the presence of 1 mM NaI. A portion of the iodinated protein was mixed with non-radioactive toxin and fragment A was isolated as described above. Toxin binding was measured by incubating mitoplasts with radioactive toxin under the conditions used for protein synthesis. After 15 min incubation, mitoplasts were pelleted and washed twice with 1 ml of Hepes sucrose medium. Samples were processed for radioactivity measurement as described above.

*Measurement of the concentration of nicotinamide adenine dinucleotide in mitoplasts*

Mitoplasts were incubated under the conditions described above for the ATPase assay, except that ATP was not added to the reaction mixture. The perchloric acid extract was neutralized to remove the Perchlorate. The total concentration of the

oxidized and reduced forms of nicotinamide adenine dinucleotide was estimated spectrophotometrically after reduction with yeast alcohol dehydrogenase (Vallee and Hoch, 1955).

## Results

*Effect of fragment A on protein synthesis in mitoplasts*

Intact mitochondria are contaminated to a variable extent by cytoplasmic ribosomes attached to the outer mitochondrial membrane and by fragments of the rough endoplasmic reticulum. Therefore, in all experiments reported here we have used mitoplasts, *i.e.* mitochondria where the outer membrane was removed by treatment with digitonin (Chan *et al.*, 1970). It has been shown that liver mitoplasts prepared in this way are free of membrane fragments from the endoplasmic reticulum and exhibit tight energy coupling (Schnaitman and Greenawalt, 1968) (see also figure 3).

The incorporation of [$^{35}$S]-methionine into protein by mitoplasts was almost linear for 30 min and thereafter declined. The time course of the reaction rules ou the possibility of bacterial contamination of the system. It is also seen that the incorporation was insensitive to cycloheximide, but sensitive to chloramphenicol, as expected for mitochondrial protein synthesis.

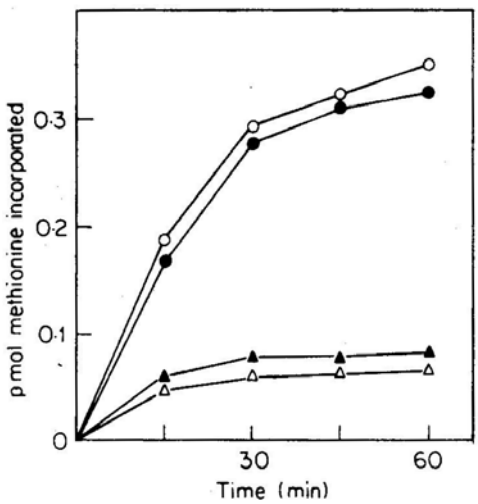


**Figure 1.** Inhibition of protein synthesis in mitoplasts by diphtheria toxin fragment A. Mitoplasts (300 µg), prepared as described under Materials and methods, were incubated for increasing periods of time at 37°C in a final volume of 50 µl. The incubation mixture contained 40 mM Hepes (pH 7.4), 70 mM KCl, 2 mM $Mg^{2+}$, 1.2 mM $Na_2$ $HPO_4$, 5 mM glutamate, 5 mM malate, 1 mM DTT, 1 mM ADP, 0.1 mM ATP, 1 µM each of 19 amino acids and 30 µCi [$^{35}$S]-methionine (specific activity 900 Ci/mmol). Inhibitors were present in the concentrations given below. At the time points indicated 5 µl samples were withdrawn into 500 µl 0.1 N NaOH, containing 1 mM methionine and 100 mM mercaptoethanol. After 3 min, protein was precipitated with 2 ml of 10% trichloroacetic acid. After heating for 30 min at 90°C, the precipitate was collected on glass fiber filters, washed 10 times with 10 ml of 10% trichloroacetic acid and counted. The radioactivity incorporated is expressed as pmol methionine/mg protein. One pmol methionine corresponds to $1.6\times10^6$ cpm. (▲), Diphtheria toxin fragment A, 200 µg/ml; (Δ), chloramphenicol, 200 µg/ml; (●), cycloheximide, 50 µg/ml; (O), control.

The interesting fact emerging from these experiments is that fragment A inhibited protein synthesis in mitoplasts to about the same extent as did chloramphenicol (figure 1). In contrast, in all experiments the complete toxin gave a slight stimulation of the amino acid incorporation. It should be noted that the concentration of fragment A, required to inhibit protein synthesis by 90% was about one order of magnitude higher in mitoplasts than in extracts of HeLa cells (Collier and Pappenheimer, 1964).

In figure 2 the inhibition of protein synthesis in mitoplasts exposed for 15 min to increasing concentrations of fragment A is shown. The dose-response relationship reveals saturation kinetics at high toxin concentration, indicating that in this system fragment A does not inactivate catalytically a component of the protein synthesizing machinery, as is the case with cytoplasmic protein synthesis.

*Inability of fragment A to inhibit protein synthesis in lyzed mitoplasts*

If the inhibition of mitochondrial protein synthesis by fragment A were the result of a catalytic inactivation of elongation factor 2, as is the case in the cytoplasm (Gill *et al.*, 1969), fragment A should inhibit protein synthesis also in lyzed mitoplasts. The protein synthesizing activity of lyzed mitoplasts was found to be rather low, compared to that of intact mitoplasts. However, in the presence of an efficient ATP regenerating system, the lyzed mitoplasts synthesized proteins at about 1/3 the

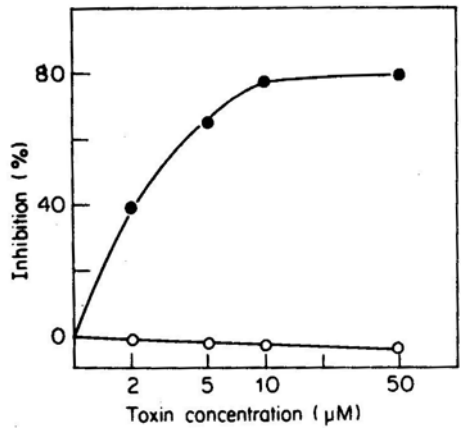


**Figure 2.** Inhibition of protein synthesis in mitoplasts pretreated with diphtheria toxin. Mitoplasts (1.6 mg), prepared as described under Materials and methods, were incubated with increasing concentrations of complete diphtheria toxin or its fragment A at 37°C for 15 min in a final volume of 200 µl. The incubation mixture contained 40 mM Hepes (pH 7.4), 70 mM KCl, 2 mM $Mg^{2+}$, 1.2 mM $Na_2HPO_4$, 5 mM glutamate, 5 mM malate, 1 mM ADP, 0.1 mM ATP and 1 mM DTT. At the end of the incubation period the reaction mixture was diluted with 1 ml of cold Hepes sucrose medium and the mitoplasts were sedimented at 2°C. The pellet was resuspended in 200 µl of fresh incubation mixture containing all the components described above, except toxin. Fifty µl of this mixture was incubated with 1 µM each of 19 amino acids and 30 µCi of [$^{35}$S]-methionine (specific activity 900 Ci/mmol) at 37°C for 40 min. Five µl samples were processed in duplicate for radioactivity measurement, as described in legend to figure 1. Control mitoplasts were treated in the same way, except that toxin was omitted. The data are expressed as per cent inhibition of protein synthesis. (●), Fragment A of diphtheria toxin; (O), complete toxin.

**Table 1.** Effect of diphtheria toxin on protein synthesis in lyzed mitoplasts.

| Conditions | [$^{35}$S]-Methionine incorporated/mg protein |
|---|---|
| | cpm×10$^{-4}$ |
| Lyzed mitoplasts | |
| No addition | 22.1 |
| Intact diphtheria toxin | 22.4 |
| Fragment A of diphtheria toxin | 22.0 |
| Cycloheximide | 20.5 |
| Chloramphenicol | 4.2 |
| Carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone | 18.9 |
| Intact mitoplasts | 68.2 |
| Fragment A of diphtheria toxin | 14.3 |
| Carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone | 16.8 |

Mitoplasts suspended in a buffer containing 5 mM Hepes (pH 7.4) and 150 mM KCl were lyzed by adding a 2% (w/v) solution of Brij 58 to a final concentration of 0.1%. After 5 min at 2°C the clear lyzed mitoplasts, equivalent to 300 µg protein, were incubated at 37°C in a buffer solution (final volume 50 µl) with the following additions: 40 mM Hepes (pH 7.4), 70 mM KCl, 2 mM $Mg^{2+}$, 1 mM ATP, 10 mM creatine phosphate, 2 units of creatine phosphokinase, 1 µM each of 19 amino acids and 30 µCi [$^{35}$S]-methionine (specific activity 900 Ci/mmol). The concentration of diphtheria toxin and fragment A, when present, was 200 µg/ml. The concentrations of cycloheximide, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and chloramphenicol were 50 µg/ml, 1.6 µg/ml and 200 µg/ml, respectively. After 40 min incubation, 5 µl samples in duplicate were processed for radioactivity measurements. The data represent the average of three experiments.

rate observed in intact mitoplasts (table 1). Also, under these conditions protein synthesis was insensitive to cycloheximide, whereas chloramphenicol inhibited the process by 80%, demonstrating that the activity measured indeed represents true mitochondrial protein synthesis. It can be seen that fragment A of diphtheria toxin did not inhibit protein synthesis in the lyzed system indicating that the inhibition observed in intact mitoplasts is caused by an indirect mechanism. Interestingly, the uncoupling agent, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone inhibited the protein synthesis in intact mitoplasts to about the same extent as did fragment A (table 1). This finding clearly suggested that the observed effect of fragment A might be due to inhibition of the energy coupling process.

*Stimulation of mitochondrial ATPase activity by fragment A*

It is well established that uncoupling of oxidative phosphorylation and other energy-dependent processes stimulate the mitochondrial ATPase activity. The effect of fragment A on the ATPase activity of mitoplasts was therefore examined. It is apparent from figure 3 that fragment A very efficiently stimulated the mitochondrial ATPase activity. In fact, fragment A was almost as effective as carbonylcyanide *p*-trifluoromethoxyphenylhydrazone in this regard (data not shown). On the other hand, intact toxin reduced slightly the ATPase activity. The results provide evidence that fragment A inhibits protein synthesis in mitoplasts by inhibiting the energy coupling process.

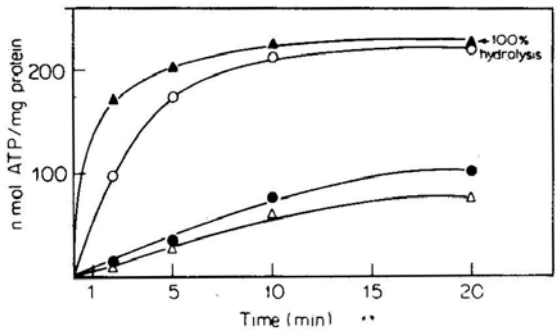


**Figure 3.** Stimulation of mitochondrial ATPase activity by fragment A of diphtheria toxin. Mitoplasts (2 mg), were incubated at 37°C for 10 min in a final volume of 200 µl. The incubation mixture contained: 40 mM Hepes (pH 7.4), 70 mM KCl, 3 mM $Mg^{2+}$, 1.2 mM $Na_2$ $HPO_4$, 5 mM glutamate, 5 mM malate and 1 mM DTT. The concentrations of complete toxin and fragment A were 2.5 mg/ml and 200 µg/ml, respectively. At the end of the incubation the reaction mixtures were diluted with 1 ml of Hepes sucrose medium and the mitoplasts were sedimented at 2°C. The pellet was resuspended in 200 µl of fresh incubation mixture containing all the components described above, except mitoplasts and toxin, and incubated with 3 mM ATP at 37°C. At the time points indicated 30 µl samples were withdrawn into 200 µl of 5% cold perchloric acid. The protein precipitate was removed by centrifugation. The supernatant was diluted three fold with 0.45 M potassium posphate, pH 3.93. Adenine nucleotides in the 20 µl aliquots of the supernatants were analyzed by high performance liquid chromatography. The chromatographic separation of the nucleotides was achieved at 20°C on a strong anion exchanger (Partisil 10-SAX), prepacked from Whatman in a 25 cm stailness steel column of 4.6 mm inner diameter. The mobile phase, consisting of 0.45 M potassium phosphate, pH 3.93, was pumped at a flow rate of 2 ml/min (1,800 p.s.i.), giving the following retention times: $t_R$ (AMP) = 2.02 min, $t_R$ (ADP) = 3.14 min and $t_R$ (ATP)= 11.70 min. The ATPase activity is expressed as nmol ATP hydrolyzed per mg protein. (●), Control; (Δ), complete toxin; (O), fragment A; (▲), control mitoplasts lyzed with 0.2% triton.

*Binding of fragment A to the mitochondrial inner membrane*

In attempts to elucidate the mechanism by which fragment A inhibits protein synthesis and stimulates ATPase activity in mitoplasts, experiments were performed to determine its binding to and possible penetration of the mitochondrial inner membrane.

From table 2 it is seen that [$^{125}$I] -labelled fragment A, as well as labelled intact toxin, bind to intact mitoplasts. The binding capacity appeared to be very similar for the two polypeptides, indicating that the stimulatory effect of fragment A on the ATPase activity cannot be explained by selective binding to the inner membrane In both cases most of the, bound radioactivity was found in the membrane fraction. Since the radioactivity present in the matrix fraction was greater after incubation with the complete toxin, which was inactive in the system, than after incubation with fragment A, entry of fragment A into the matrix is apparently not required for the inhibition of the mitochondrial protein synthesis.

*Role of enzymatic activity of fragment A*

Since fragment A has $NAD^+$-glycohydrolase activity (Collier, 1975), its effect on the intramitochondrial concentration of $NAD^+$ and NADH was measured. It is

**Table 2.** Binding of radioactive diphtheria toxin and fragment A to mitoplasts.

| Additions | Radioactivity bound/mg protein | | |
|---|---|---|---|
| | Intact mitoplast | Membrane fraction | Matrix fraction |
| | cpm | cpm | cpm |
| Complete toxin | 5,860 | 10,200 | 1,560 |
| Fragment A | 4,950 | 10,800 | 502 |

[$^{125}$I]-Labelled diphtheria toxin ($1\times10^5$cpm), and fragment A ($1\times10^5$cpm) were incubated for 15 min at 37°C with 3 mg mitoplasts in a final volume of 200 µl under the conditions used for protein synthesis (legend to figure 1), except that labelled methionine was replaced by non-radioactive methionine. Mitoplasts were pelleted at 2°C and washed twice with 1 ml of Hepes sucrose medium. Membrane proteins and matrix proteins were separated by centrifugation of sonicated mitoplasts at 100,000*g* for 1 h. The pellet of intact mitoplasts and membrane fraction was dissolved in 200 µl of 0.1 N NaOH. Twenty µl of dissolved protein and supernatant fraction was precipitated in duplicate with 10% trichloroacetic acid. The precipitate was collected on glass fiber filters for radioactivity measurement.

**Table 3.** Estimation of the concentration of pyridine nucleotides in mitoplasts exposed to fragment A.

| Treatment | Total pyridine nucleotides |
|---|---|
| | nmol · mg$^{-1}$ protein |
| None | 12.1 |
| Fragment A | 11.8 |

Mitoplasts (4.2 mg) were incubated at 37°C for 15 min in a final volume of 400 µl. The incubation mixture contained: 40 mM Hepes (pH 7.4), 50 mM KCl, 2 mM $Mg^{2+}$, 1.2 mM $Na_2HPO_4$, 5 mM glutamate, 5 mM malate and 1 mM DTT. The concentration of fragment A, when present, was 200 µg/ml. The reaction was stopped by the addition of 2 ml of cold 5% perchloric acid. After 15 min at 2°C, the mixture was neutralized with cold 1 M potassium bicarbonate and the precipitate was removed by centrifugation. The supernatant was concentrated, and the total pyridine nucleotide concentration was estimated after reduction with alcohol dehydrogenase in presence of ethanol. The concentrated nucleotide fraction was incubated for 30 min at 25°C in a final volume of 1 ml. The incubation mixture contained 20 mM glycine buffer (pH 8.7), 75 mM sodium phosphate, 10 µl ethanol, 75 mM semicarbazide (pH 8.7) and 10 µg yeast alcohol dehydrogenase (specific activity 150 units/mg). At the end of the incubation, the absorption at 340 nm was measured and the concentration of NADH was calculated, using the molar extinction coefficient $\varepsilon = 6.22\times10^3$. The results represent the average of two experiments.

seen from table 3 that the level of $NAD^+$ in the mitoplasts did not change when they were incubated with fragment A at concentrations that efficiently inhibited protein synthesis and stimulated ATPase activity. The data are consistent with the above finding (table 2) that most of fragment A binds to mitoplasts and can be recovered in the isolated inner membrane fraction.

To study the possibility that fragment A might exert its effect on mitochondrial protein synthesis by catalyzing ADP-ribosylation of mitochondrial proteins, we incubated intact mitoplasts and their subfractions with [$^{14}$C]-labelled $NAD^+$, using liver cytosol as control. From table 4 it is seen that no ADP-ribosylation of mitochondrial proteins was detected.

**Table 4.** Ability of diphtheria toxin fragment A to ADP-ribosylate proteins from different subcellular fractions.

| Additions | Diphtheria toxin fragment A | Radioactivity incorporated |
|---|---|---|
| | | cpm |
| Cytosol | + | 1145 |
| | − | 39 |
| Nuclear fraction | + | 204 |
| | − | 212 |
| Intact mitoplast | + | 38 |
| | − | 26 |
| Membrane fraction | + | 23 |
| Matrix fraction | + | 28 |
| Membrane + matrix | + | 32 |

Mitoplasts (7 mg) were lyzed by sonication and the suspension was centrifuged for 1 h at 10,000 *g* to prepare the membrane (pellet) and the matrix (supernatant) fractions. The membrane fraction was resuspended in 200 µl buffer (50 raM Tris-HCl, pH 8, 70 mM DTT and 0.1 mg/ml bovine serum albumin). The supernatant as well as the membrane fraction were dialyzed against 500 ml of the buffer for 3 h. The nuclear fraction from mouse liver was lyzed with 0.2% sodium deoxycholate. Lyzed nuclear fraction and cytosol fraction from mouse liver were dialyzed against the buffer before use. Each fraction, containg 200 µg of protein, was incubated with 20 µl of [$^{14}$C]-$NAD^+$, labelled in the adenosine moiety, and 10 µg of diphtheria toxin fragment A at 37°C in a final volume of 100 µl. Twenty µl samples were withdrawn in duplicates at 0 min and 10 min, precipitated and washed with 5% trichloroacetic acid for radioactivity measurements. The 0-min values were subtracted from each sample. In the sample containing both mitoplast membrane and matrix protein, the total protein concentration in the reaction mixture was 200 µg. The results represent the average of 5 experiments.

## Discussion

The present finding that fragment A of diphtheria toxin efficiently inhibits protein synthesis in mitoplasts from mouse liver was unexpected since the intact toxin is unable to inhibit protein synthesis in mitoplasts (figure 2) as well as in yeast mito-

chondria and prokaryotes (Richter and Lipmann, 1970). The mechanism of the inhibition is, however different from that operating in the cytoplasm where fragment A inhibits elongation factor 2 by ADP ribosylation (Gill *et al.*,1969). This follows from the findings that fragment A had no inhibitory effect on the protein synthesis in lyzed mitoplasts containing an ATP regenerating system (table 1) and that no $NAD^+$-glycohydrolase activity or ADP ribosylation of mitochondrial proteins could be detected (tables 3 and 4). It is clear that fragment A has no direct effect on the mitochondrial protein synthesis as such and that the effect of protein synthesis in mitoplasts is brought about by an indirect mechanism. The ability of fragment A to inhibit protein synthesis in intact, but not in lyzed mitoplasts may be used as an additional criterion of specificity for mitochondrial protein synthesis.

In the present study the [$^{35}$S]-methionine incorporation into acid-insoluble material was assayed in the presence of added external adenine nucleotides and under maximally energized conditions, where mitochondrial respiration was supported by NADH-linked substrates. Previously Wheeldon and Lehninger (1966) have observed that uncouplers of oxidative phosphorylation efficiently inhibit respiration-dependent mitochondrial protein synthesis. In agreement with this it was found that carbonylcyanide *p*-trifluoromethoxyphenylhydrazone inhibited protein synthesis in mitoplasts as efficiently as did fragment A. The present results can therefore be explained by an uncoupling effect of diphtheria toxin fragment A Direct evidence that fragment A indeed uncouples oxidative phosphorylation, was given in figure 3 where it was shown that fragment A stimulates strongly the ATPase activity of mitoplasts. The finding that after sonication, labelled fragment A could be almost completely recovered from the membrane fraction (table 2), is consistent with a tight binding of this peptide to the mitochondrial inner membrane.

In view of the fact that most diphtheria toxin preparations contain some nicked toxin which may dissociate to give fragment A, it may seem surprising that diphtheria toxin had no inhibiting effect on protein synthesis in the mitoplasts. One possible explanation is, that in the present incubation system, it was necessary to keep the concentration of reducing agent (DDT) below 2 mM, to avoid inhibition of protein synthesis. Conceivably, the low concentration of DDT used was insufficient to release fragment A from nicked toxin molecules.

The question arises, whether the uncoupling effect of fragment A may play a role in the intoxication of cells by diphtheria toxin. This seems unlikely in view of the fact that fragment A inhibits cellular protein synthesis by a catalytic mechanism. Thus, the presence in the cytosol of a single molecule of diphtheria toxin is sufficient to kill cells in culture (Pinchot, 1950). In contrast, the uncoupling effect of fragment A is non-catalytic. Therefore, since each cell contains numerous mitochondria, a large number of molecules is required to inhibit significantly oxidative phosphorylation. Nevertheless, it cannot be ruled out that, in intact animals treated with large doses of diphtheria toxin, the effect of fragment A on oxidative phosphorylation may also be expressed before the animal succumbs to the protein synthesis inhibition. In fact, it has been reported that increased oxygen consumption and reduced levels of high energy phosphate compounds may be observed in mitochondria isolated from the liver and kidneys of pigs treated with intact diphtheria toxin (Pinchot, 1950; Niselovskaya and Paderina, 1978).

Irrespective of its possible role in intoxication of cells, the ability of fragment A to uncouple phosphorylation seems to be of considerable interest. It is known that the linear oligopeptide, gramicidin, uncouples oxidative phosphorylation (Hotchkiss, 1944) by forming ion conducting channels when inserted into the mitochondrial inner membrane (Moore and Pressman, 1964). Whether fragment A acts by a similar mechanism is not evident from the present data. It can be said with reasonable certainty that the uncoupling effect of fragment A is unrelated to its well known enzymatic activity. It seems likely that fragment A acts by altering the properties of the mitochondrial inner membrane. Clearly, further work is needed to elucidate its mechanism of action.

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