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Phosphorylation and Hydrolysis of 7-Deazaadenine Nucleotides by Rat Liver and Beef Heart Mitochondria†

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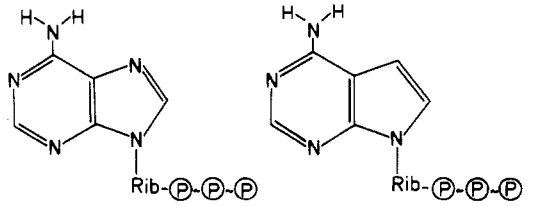
ABSTRACT: Tubercidin nucleotides [tubercidin 5′-monophosphate (TuMP), 5′-diphosphate (TuDP), and 5′-triphosphate (TuTP)] were tested as potential substrates for the mitochondrial phosphotransferases from rat liver and beef heart. TuDP is recognized by the mitochondrial translocase and phosphorylated by the respiratory chain enzymes in both mitochondria and submitochondrial particles from rat liver and beef heart; the low transport rate of the analogue into the matrix space of the intact organelles seems to be not a limiting step in the formation of TuTP. The phosphorylation of TuDP is significantly lower in beef heart mitochondria because of a higher specificity for ADP of the heart oxidative phosphorylation system. On the basis of the kinetic parameters of the partially purified liver mitochondrial adenylate kinase, one can conclude that the liver mitochondria are able to phosphorylate in vivo TuMP at a rate practically equal to the rate of AMP phosphorylation. The liver mitochondrial NDP kinase ensures a further fast phosphorylation of TuDP without the direct involvement of respiratory chain enzymes. In the case of heart mitochondria, two factors limit the rate of TuMP phosphorylation to TuTP: the lower acceptor activity of adenylate kinase with TuMP as compared with AMP and the different localization of heart NDP kinase situated on the inner face of the inner mitochondrial membrane. TuDP and TuTP preserve the ability of the natural nucleotides to interact with the "tight" nucleotide binding sites of isolated or membrane-bound F_1. The low hydrolytic rate of TuTP with F_1 may be related to the unusual flexibility of the glycosyl bond of tubercidin nucleotides in aqueous solution, with a high accessibility to syn conformation.

Tubercidin[1] is a highly cytotoxic nucleoside to microbial as well as to mammalian cells (Acs et al., 1964; Nishimura et al., 1966; Bloch et al., 1967). This compound is not a substrate for adenosine deaminase (Agarwal et al., 1975), and it is rapidly phosphorylated to TuTP which may substitute for ATP in a large variety of reactions (Henderson & Khoo, 1965; Smith et al., 1970; Suhadolnik, 1970; Bhuyan et al., 1971; Weiss & Pitot, 1974; Seela et al., 1981). However, despite the great diversity of its actions, the mechanism responsible for inhibition of growth and metabolism of cells is still obscure.

In the present study the role of various compartments of rat liver and beef heart mitochondria in phosphorylation and hydrolysis of 7-deazaadenine nucleotides is described. Our interest was aroused by the fact that participation of 7-deazaadenine nucleotides in mitochondrial reactions of phosphoryl group transfer may offer a better explanation for the cytotoxic effects of tubercidin on eucaryotic cells (Bisel et al., 1970; Grage et al., 1970; Ross & Jaffe, 1972; Bloch, 1975). On the other hand, the close structural resemblance of both adenine and 7-deazaadenine (Chart I) might provide more

Chart I: Structure of ATP (a) and TuTP (b)[a]



a. b.

[a] Rib-P-P-P = ribosyl 5′-triphosphate.

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[1] Abbreviations: tubercidin, 4-amino-7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]pyrimidine or 7-deazaadenosine; TuMP, TuDP, and TuTP, tubercidin 5′-mono-, 5′-di-, and 5′-triphosphate; 8-BrIDP, 8-bromoinosine 5′-diphosphate; o^{1}ADP and o^{1}ATP, adenosine N^1-oxide 5′-di- and 5′-triphosphate; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; AMP-P-(NH)P, 5′-adenylyl β,γ-imidodiphosphate; NDP and NTP, nucleoside 5′-di- and 5′-triphosphate; NDP kinase, nucleosidediphosphate kinase (EC 2.7.4.6); G6P, glucose 6-phosphate; 6PG, 6-phosphogluconic acid; F6P, fructose 6-phosphate; FDP, fructose 1,6-bisphosphate; MA, MgATP submitochondrial particles; MAU, MgATP submitochondrial particles treated with urea; FCCP, carbonyl cyanide (trifluoromethoxy)-phenylhydrazone; EDTA, ethylenediaminetetraacetic acid; Tris, tris-(hydroxymethyl)aminomethane.

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subtle information on the significance of the purine moiety of ATP and ADP in determining the specificity of mitochondrial processes involving translocation and oxidative phosphorylation (Hohnadel & Cooper, 1972a; Schlimme et al., 1973; Kauffman et al., 1978; Harris et al., 1978; Lascu et al., 1979; Boos & Schlimme, 1979).

Materials and Methods

Chemicals. All purine nucleotides (except dADP), nicotinamide adenine nucleotides, substrates, and coupling enzymes were commercial products from Boehringer Mannheim. Tubercidin and dADP were products of Sigma Chemical Co. [^{32}P]Orthophosphate was obtained from the Institute of Physics and Nuclear Engineering, Bucharest. [^{14}C]ADP was purchased from the Radiochemical Centre, Amersham. TuMP was prepared from unblocked nucleoside by the method of Yoshikawa et al. (1967). TuDP was obtained from monophosphate by the method of Hoard & Ott (1965). TuTP was prepared enzymatically from TuDP and excess of phosphoenolpyruvate and pyruvate kinase. Other nucleoside and nucleotide analogues were synthesized according to previously published procedures (Mantsch et al., 1975; Bârzu et al., 1976a; Lascu et al., 1979). Commercial samples of dADP, dATP, AMP-P(NH)P, Ap_5A, GDP, GTP, and $NADP^+$ were purified by chromatography on DEAE-Sephadex A-25.

Biochemical Assays. The isolation of rat liver mitochondria, the measurement of mitochondrial respiration, and the exchange of intramitochondrial ^{14}C-labeled adenine nucleotides with externally added nucleotide analogues were performed as described in previous publications (Jebeleanu et al., 1974; Mantsch et al., 1975; Bârzu et al., 1976b). Heavy beef heart mitochondria were prepared as described by Löw & Vallin (1963), stored for 2–7 days at −10 °C, and thawed only shortly before use. MgATP submitochondrial particles (MA particles) were prepared according to Löw & Vallin (1963). MA particles were depleted of F_1 by treatment with urea (Racker & Horstman, 1967) as described by Tuena de Gômez-Puyou et al. (1979). MA particles (50 mg of protein in 2.5 mL of 0.25 M sucrose) were diluted with 2.5 mL of freshly prepared 4 M urea, 4 mM EDTA, and 100 mM Tris–sulfate (pH 8). The mixture was incubated in the cold for 40 min and thereafter centrifuged at 150000*g* for 20 min. The pellet was suspended in 5 mL of 0.25 M sucrose, centrifuged again for 20 min at 150000*g*, and finally resuspended in 0.25 M sucrose at 25–30 mg of protein mL^{-1}. This preparation is referred to as MAU particles. Rat liver submitochondrial particles were obtained as described by Kielley (1963). F_1-ATPase was prepared from rat liver and beef heart submitochondrial particles by using the chloroform extraction procedure (Beechey et al., 1975) as described by Spitsberg et al. (1978) and Penin et al. (1979), respectively, and stored at 0–4 °C as an ammonium sulfate suspension supplemented with 1 mM EDTA and 4 mM ATP. Removal of adenine nucleotides from beef heart F_1 was done by using the procedure described by Garrett & Penefsky (1975). The ATP + ADP content of ammonium sulfate washed F_1 (the "tightly bound" nucleotides), of nucleotide-depleted F_1, and of submitochondrial particles was measured by using a "cyclic" assay system (Petrescu et al., 1981). Samples containing 1–5 nmol of ATP + ADP (0.5 mg of purified F_1 or 2–5 mg of MA or MAU particles) in 0.2 mL were treated with 0.8 mL of 1 M perchloric acid and centrifuged for 10 min at 5000*g*. The KOH-neutralized extract (0.1–0.3 mL) was added directly into the test system containing, in 1-mL final volume, 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 5 mM creatine phosphate, 10 mM glucose, 0.4 mM $NADP^+$, and 5 units of

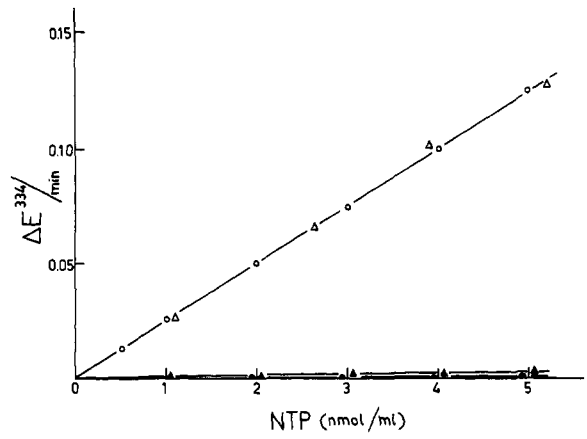

FIGURE 1: Relationship between the rate of NADPH formation measured at 334 nm and NTP concentration. (○) ATP, (△) TuTP, (▲) dATP, and (●) GTP.

creatine kinase. The reaction was started with 1.5 units of hexokinase and 1.8 units of glucose-6-phosphate dehydrogenase, and the absorption increase at 334 nm was recorded for several minutes. Since the above described procedure is sensitive to pH variations, taking advantage of the fact that ΔE/min is additive to additions of ADP or ATP, an internal standard was used for each determination, consisting of two successive injections of 1 nmol of ADP or ATP. The main advantages of the "cyclic" determination are precision, sensitivity, and specificity, within the normal photometric routine. Of the analogues investigated, only TuDP + TuTP shows properties similar to those of adenine nucleotides (Figure 1).

All enzymatic rates were determined at 25 °C in a 1-mL final volume. When the activity was measured in systems involving the interconversion of pyridine nucleotides, an Eppendorf 1101 M photometer equipped with a W+W 4410 type recorder (full scale deflection 0.25 absorbance unit) was used. Phosphorylation of ADP and its analogues by respiratory chain enzymes from intact rat liver and beef heart mitochondria was followed in a reaction medium containing 180 mM sucrose, 50 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 2 mg of defatted bovine serum albumin, 5 mM glutamate + 5 mM malate, 2 mM $^{32}P_i$ (200 cpm/nmol), and 0.6 mM nucleoside diphosphate. The reaction was initiated by addition of 0.4–1.2 mg of mitochondrial protein. After incubation for 5 (ADP) or 10 min (dADP and TuDP) 1 mL of 1 M perchloric acid was added, and the KOH-neutralized extract was subjected to column chromatography (0.55 × 50 cm) on DEAE-Sephadex A-25, using a linear gradient from 0 to 1.5 M sodium acetate in 0.05 M Tris–acetate buffer (pH 7) at a flow rate of 20 mL/h. Fractions (between 0.005 and 0.200 mL) corresponding to the radioactive peaks were used for radioactivity counting (ABAC SL 40 liquid scintillation spectrometer, Intertechnique, France). Oxidative phosphorylation in submitochondrial particles was measured in a medium containing 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 2–4 mM $^{32}P_i$ (200 cpm/nmol), 0.5 mM EDTA, 2.5 mM $MgCl_2$, 2 mg of defatted bovine serum albumin, 5 mM glucose, 5 units of hexokinase, 0.1 mM Ap_5A, 0.5 mM $NADP^+$, 1.8 units of glucose-6-phosphate dehydrogenase, 1 μg of rotenone, and particles (0.3–0.5 mg of protein). After 2 min of preincubation, the reaction was started by addition of 5 mM succinate and 0.2 mM NDP. After 10 min of incubation, the reaction was stopped with perchloric acid, and the neutralized extract was subjected to column chromatography to separate and quantify the [^{32}P]6PG. The ATP-dependent NAD^+ reduction by succinate catalyzed by beef heart MA particles was

Table I: Exchange of Intramitochondrial ^{14}C-Labeled ADP with Extramitochondrial TuDP and dADP[a]

additions	[^{14}C]ADP exchanged (%)
ADP	100
ADP + 0.05 mM atractyloside	7.6
TuDP	4.2
TuDP + 0.05 mM atractyloside	<1
TuDP + ADP	107
dADP	19
dADP + 0.05 mM atractyloside	3.4

[a] Rat liver mitochondria (4–5 mg of protein) which had been loaded previously with [^{14}C]ADP were incubated for 10 min at 2 °C in 110 mM KCl, 20 mM Tris-HCl (pH 7.4), and 1 mM EDTA at a final volume of 0.4 mL. The reaction was triggered by addition of 0.2 mM ADP, TuDP, or dADP. The incubation was stopped by addition of 0.2 mM atractyloside; after centrifugation at 20000*g* for 5 min, 0.2-mL aliquots of the supernatant were used for radioactivity counting.

measured in a reaction mixture containing 200 mM sucrose, 50 mM KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM NAD^+, 1 mM KCN, 10 mM succinate, and 0.1–0.2 mg of protein of MA particles. After 2–3 min of preincubation, the reaction was started by addition of NTP. The fluorescence increase due to NADH formation was measured with an Eppendorf fluorometer using the appropriate filter combinations (excitation wavelength 366 nm; emission wavelength 470–3000 nm). The ATP-dependent transhydrogenase reaction was measured in a medium containing 250 mM sucrose, 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 100 mM ethanol, 60 units of alcohol dehydrogenase, 1 μg of antimycin A, and 0.5–0.7 mg of submitochondrial particles. After 2–3 min of preincubation, 0.02 mM NAD^+, 0.25 mM $NADP^+$, and 0.6 mM NTP were added to trigger the reaction. ATPase activity of soluble or membrane-bound F_1 was measured both by following the release of P_i from NTP or by using a regenerative system. In the first case the reaction medium contained 100 mM KCl, 20 mM Tris-HCl (pH 8), 2.5 mM $MgCl_2$, 0.5 mM EDTA, and 1.5 mM NTP. The reaction was initiated by addition of 0.04–0.08 mg of submitochondrial particles or 0.3–10 μg of purified F_1. After 2 (ATP and dATP) or 10 min (TuTP and GTP) of incubation, the reaction was stopped with 0.5 mL of 10% trichloroacetic acid, and released P_i was measured colorimetrically by the method of Lowry & Lopez (1946). In the second case the medium contained 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8), 0.15 mM NADH, 1 mM phosphoenolpyruvate, 2–10 units of pyruvate kinase, 2 units of lactate dehydrogenase, and 0.3 mM NTP. The reaction was initiated by addition of enzyme. Proteins were estimated by the method of Lowry et al. (1951). Experimental details, if different from those given here, are mentioned in the legend to the figures and the tables.

Results

Exchange of Rat Liver Intramitochondrial ^{14}C-Labeled ADP with Extramitochondrial NDPs. Table I shows that the exchange between internal [^{14}C]ADP and externally added TuDP and dADP is 4% and 19%, respectively, as compared to the exchange of ADP. Both TuDP and dADP exchanges are atractyloside sensitive as shown by Duée & Vignais (1969) for the latter nucleotide. It is evident that the mitochondrial translocase shows a high degree of specificity toward the heterocyclic component of ADP and ATP. Except the formicin and 3′-deoxyadenosine nucleotides (Graue & Klingenberg, 1979; Boos & Schlimme, 1979), none of the analogues modified in the purine or ribose ring is recognized by the

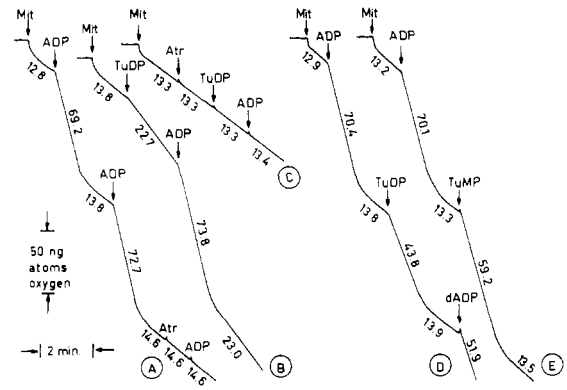

FIGURE 2: Effect of TuDP, TuMP, and dADP on the respiratory rate of rat liver mitochondria. The respiratory medium contained the following at 0.5-mL final volume and 22 °C: 180 mM sucrose, 50 mM KCl, 25 mM Tris-HCl (pH 7.4), 10 mM KP_i (pH 7.4), 0.5 mM EDTA, 2.5 mM $MgCl_2$ (only in experiments shown by traces D and E), 1 mg of bovine serum albumin, 5 mM glutamate, and 5 mM malate. The reaction was initiated by addition of 1.3 mg of mitochondrial protein. At the points indicated, 0.2 μmol ADP, 0.3 μmol (traces B and C) or 0.15 μmol (trace D) TuDP, 0.2 μmol dADP, 0.08 μmol TuMP, and 0.025 μmol atractyloside were added. The numbers beside the traces indicate the oxygen consumption expressed as ng-atom min^{-1} (mg of protein)$^{-1}$.

mitochondrial translocase (Duée & Vignais, 1969; Jebeleanu et al., 1974; Schlimme et al., 1977; Kauffman et al., 1978; Lascu et al., 1979).

Effect of 7-Deazaadenine Nucleotides on Mitochondrial Oxygen Consumption. The addition of 0.6 mM TuDP to intact rat liver mitochondria, both in the absence of Mg^{2+} (trace B in Figure 2) or in the presence of Mg^{2+} (not shown), led to a stimulation by about 60% of the rate of oxygen consumption. This effect is sensitive to atractyloside. Of all the other NDP tested with this respect, only dADP had a similar effect. Addition of TuDP (as well as of dADP or TuMP, but not dAMP) to respiring rat liver mitochondria, after the state 3 to state 4 transition with state 3 being induced by 0.4 mM ADP, does result in an appreciable stimulation of the rate of respiration, which is strongly dependent on the Mg^{2+} concentration in the medium (no stimulation occurs in the absence of the cation) as well as on the analogue concentration itself. These results can be explained as follows: Stimulation of the oxygen consumption by both TuDP and dADP, even in the absence of Mg^{2+} in the medium, is due to the phosphorylation of these nucleotides by the enzymes of oxidative phosphorylation (the matrix space contains enough Mg^{2+} to support NDP phosphorylation). The stimulation of mitochondrial respiration by addition of TuDP, dADP, and TuMP after the state 3 to state 4 transition (Figure 2, trace D and E) is due to the coupling of the respiratory chain linked ADP phosphorylation to the reaction catalyzed by NDP kinase and adenylate kinase, respectively, on the outer side of the inner mitochondrial membrane. TuMP, unlike other nucleoside monophosphates, including dAMP, serves as a substrate for adenylate kinase. The ratio TuMP/O approaching 1 when succinate was used as respiratory substrate proved the high "efficiency" of the NDP kinase + adenylate kinase + TuMP trapping system.

Unlike the rat liver mitochondria, the beef heart mitochondria do not show a significant stimulation of oxygen consumption in the presence of TuDP or TuMP regardless of the presence or absence of magnesium ions.

Incorporation of $^{32}P_i$ in NTP Catalyzed by Rat Liver and Beef Heart Mitochondria. Since measurements of the oxygen consumption offers only indirect information on the phosphorylation of TuDP by enzymes of the respiratory chain, the

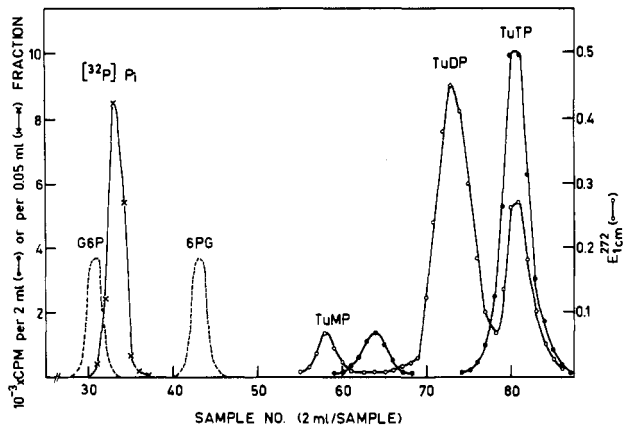

FIGURE 3: Phosphorylation of TuDP by respiratory chain enzymes from rat liver mitochondria. The experimental conditions were described under Materials and Methods. $^{32}P_i$ and TuDP concentrations were 4 mM and 0.6 mM, respectively. The reaction was initiated by addition of 1.06 mg of mitochondrial protein; incubation time 10 min. For the estimation of TuTP and TuDP concentration, the value of 10.6 for ϵ_{mM} at 272 nm was taken. The interrupted lines indicate the elution intervals of standard solutions containing G6P and 6PG obtained in other series of experiments.

Table II: Phosphorylation of TuDP and of Other Nucleoside Diphosphates by Respiratory Chain Enzymes from Intact Rat Liver and Beef Heart Mitochondria[a]

	[^{32}P]NTP [nmol min^{-1} (mg of protein)$^{-1}$]			
	rat liver		beef heart	
nucleotide	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
ADP	134	130	109	112
TuDP	20.3	27.4	2.5	2.6
dADP	28.1	34.3	13.5	13.2
o^{1}ADP	0	5.7		

[a] The experimental conditions were described under Materials and Methods. Mg^{2+} when present was 2.5 mM. The results are the mean of three separate experiments.

$^{32}P_i$ incorporation into NTP was followed in another series of experiments. Mitochondria were incubated with substrates, NDP, and $^{32}P_i$ either in the presence or in the absence of Mg^{2+} (to inhibit both NDP kinase and adenylate kinase reaction). The reaction mixture was then subjected to chromatographic separation to identify and quantify the individual products. The peaks corresponding to different nucleotides were located both by the elution volume (using appropriate standards) and by their UV absorption spectra (Figure 3). The specific radioactivity of ^{32}P incorporated into NTP was in a ratio of 1:1 with that of $^{32}P_i$ (between 193 and 211 cpm/nmol of NTP). These experiments demonstrated unequivocally the participation of TuDP and dADP as substrates of the oxidative phosphorylation catalyzed by rat liver and beef heart mitochondria under conditions in which all other mitochondrial reactions involving transfer of phosphoryl group are inhibited (Table II). In fact, this is only possible on intact mitochondria in the absence of exogenous Mg^{2+}, which limits such experiments to those analogues which are transported across the inner mitochondrial membrane. In the presence of Mg^{2+}, the phosphorylation of TuDP and dADP by rat liver mitochondria increases significantly (with 35% and 22%, respectively), due to the intervention of the mitochondrial NDP kinase. It is worth mentioning that o^{1}ADP, a nucleotide which neither is translocated nor serves as a substrate in oxidative phosphorylation (Mantsch et al., 1975; Bârzu et al., 1976b) is phosphorylated by rat liver mitochondria in the presence of Mg^{2+} at a rate of 5.7 nmol min^{-1} (mg of protein)$^{-1}$ by coupling the intramitochondrial ADP phosphorylation to the NDP kinase reaction. The phosphorylation of TuDP and dADP in beef heart mitochondria is less intense than in the case of rat liver, either because of a smaller translocation rate or because of a higher specificity for ADP of the heart system of oxidative phosphorylation.

When rat liver mitochondria are incubated with ADP or TuDP in the presence of Mg^{2+}, there is one other radioactivity peak, accompanying the NDP band, which results from the following reactions:

$$NDP + {}^{32}P_i \xrightarrow{\text{respiratory chain}} [{}^{32}P]NTP$$

$$2NDP \overset{AK}{\rightleftharpoons} NMP + NTP$$

$$NMP + [{}^{32}P]NTP \overset{AK}{\rightleftharpoons} [{}^{32}P]NDP + NDP$$

Finally it deserves mentioning that with rat liver mitochondria (but not with submitochondrial particles or beef heart mitochondria) both in the presence or in the absence of Mg^{2+}, with glutamate and malate as respiratory substrates (but not with succinate), a constant radioactive peak (the unlabeled peak in Figure 3) was obtained regardless of the nature of the NDP. This compound (probably phosphoenolpyruvic acid) is formed at a rate of 1.5–2.4 nmol min^{-1} (mg of protein)$^{-1}$.

Phosphorylation of TuDP by Submitochondrial Particles from Rat Liver and Beef Heart. From phosphorylation experiments on intact mitochondria, it is not possible to establish whether the limiting factor is the translocation step or the enzymatic process itself. In principle an answer to this may be obtained from measurements of oxidative phosphorylation on submitochondrial particles. However, such experiments do not allow one to follow directly the formation of NTP. Instead one can follow P_i incorporation into G6P or 6PG by using a trapping system with glucose + hexokinase. A limiting factor in the correct estimation of the oxidative phosphorylation on submitochondrial particles is the high specificity of yeast hexokinase for nucleotides as substrates (Hohnadel & Cooper, 1972b). When ADP analogues are tested, a large excess of hexokinase is thus required. One other yet unexplored possibility is a different trapping system consisting of F6P + phosphofructokinase, which has the following advantages: (a) Phosphofructokinase has a broad specificity for NTP and high affinity for both substrates expressed by K_m values below 0.1 mM (Bârzu et al., 1977). (b) Neither F6P nor FDP is hydrolyzed by the mitochondrial ATPase. (c) [^{32}P]FDP may conveniently be separated from $^{32}P_i$ by chromatography on DEAE-Sephadex A-25. In Table III are shown results obtained on the phosphorylation of TuDP, ADP, and dADP by submitochondrial particles using both trapping systems. The two systems give identical results with TuDP. In the case of ADP, the glucose + hexokinase system proved to be superior, whereas the F6P + phosphofructokinase system was better in the case of dADP. Regardless of the analytic system employed, the results on the submitochondrial particles lead to the same conclusions: (a) The phosphorylation of TuDP is significantly lower on particles from beef heart than from rat liver mitochondria. (b) The transport of TuDP and dADP into the matrix space of mitochondria of the two tissues investigated is not a limiting step in their phosphorylation to the corresponding NTPs. (c) Our values for dADP phosphorylation in beef heart submitochondrial particles are significantly smaller than those reported by Harris et al. (1978).

Hydrolysis of TuTP and Its Effect on the Energy-Requiring Reactions. As shown in Table IV and Figure 4, in view of the relative lack of specificity of mitochondrial ATPase for NTP (Schuster et al., 1975, 1976; Harris et al., 1978; Lascu

Table III: Synthesis of G6P, FDP, [^{32}P]6PG, or [^{32}P]FDP by Submitochondrial Particles in the Presence of ADP Analogues and Glucose + Hexokinase or F6P + Phosphofructokinase as Trapping Systems[a]

	phosphorylation rate [nmol min^{-1} (mg of protein)$^{-1}$]			
	rat liver		beef heart	
nucleotide	G6P	FDP	[^{32}P]6PG	[^{32}P]FDP
none	4.9	5.5	3.4	3.7
ADP	90.3	86.3	71.7	66.3
TuDP	16.8	17.1	6.0	6.6
dADP	20.5	25.2	11.4	14.5

[a] After incubation of submitochondrial particles with succinate, NDP, $^{32}P_i$, and glucose + hexokinase (±NADP$^+$ and glucose-6-phosphate dehydrogenase) or F6P (2 mM) + phosphofructokinase (2 units) as described under Materials and Methods, the reaction was stopped with 1 M perchloric acid. The KOH-neutralized extract was subjected either to chromatographic separation and measurement of radioactive compounds or to enzymatic assay for G6P or FDP content. The results are the mean of two separate experiments.

Table IV: Nucleotide Specificity of NTP Hydrolysis, of Energy-Linked Reduction of NAD$^+$ by Succinate, and of Transhydrogenase Reaction Catalyzed by Rat Liver and Beef Heart Submitochondrial Particles[a]

	rat liver		beef heart	
nucleotide	ATPase	transhydrogenase	ATPase	NAD$^+$ reduction
none		3.7		
ATP	1560 (100)	20.3 (100)	1360 (100)	42.2 (100)
TuTP	80 (5)	3.8 (<1)	35 (2.6)	0 (0)
dATP	1480 (95)	13.1 (57)	1280 (94)	14.5 (34)
GTP	370 (24)	8.1 (27)	204 (15)	0 (0)

[a] The ATPase reaction was measured colorimetrically by following the release of P_i from NTP. The transhydrogenase reaction and the NAD$^+$ reduction were started with 0.6 mM NTP. The enzymatic rates were expressed as nmol min^{-1} (mg of protein)$^{-1}$. The figures in brackets represent relative activities. The non-energy transhydrogenase activity was substracted from each sample before calculating the percent activity of the individual nucleotides (ATP = 100%).

et al., 1979), the hydrolysis of TuTP by soluble or membrane-bound F_1 was unexpectedly low as compared to the hydrolysis of ATP or dATP. Comparing the effects of various cations on the hydrolytic activity of beef heart F_1, we found that the preferences ranked the same ($Mg^{2+} > Mn^{2+} > Cd^{2+} > Ca^{2+}$) regardless of whether ATP, dATP, or GTP was used as substrate. In the case of TuTP, the maximum hydrolytic activity was obtained in the presence of Mn^{2+}, which slightly exceeded (by 25%) that corresponding to Mg^{2+}. Preincubation of soluble F_1 with 1 mM TuDP inhibited the hydrolytic activity by 35%, an effect which approached that obtained with ADP (Harris et al., 1978; DiPietro et al., 1980). The inhibition with an equivalent concentration of ADP was 58%. When F_1 was incubated in the presence of 10-fold lower concentrations of NDP, the ATPase activity was inhibited by 10% with TuDP and 32% with ADP, respectively. Kinetic studies on purified F_1 from rat liver and beef heart mitochondria revealed some peculiarities determined by the nature of NTP. At 25 °C and low substrate concentrations, the ATP hydrolysis displayed an evident biphasic character, typical for hysteretic enzymes (Frieden, 1970, 1979). The observed effect was not due to the reaction product (ADP) since the measurements were performed in a regenerative system. The hydrolytic activity of F_1 with any other analogue was linear for at least 10 min (Figure 4). On the other hand, the $1/v$ vs. $1/[NTP]$ plot was

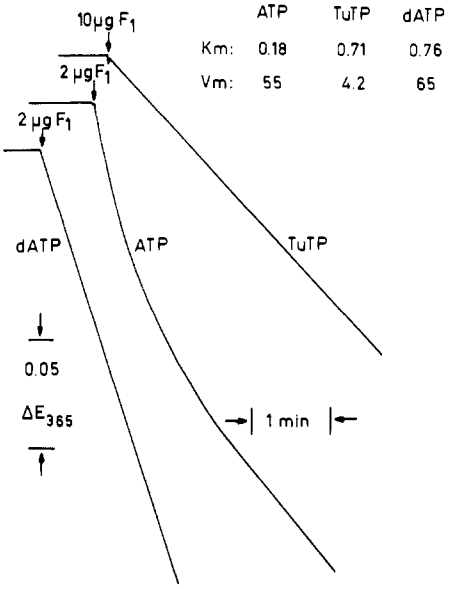

FIGURE 4: Hydrolysis of ATP, TuTP, and dADP by purified rat liver mitochondrial F_1-ATPase. The regenerative assay system with 0.3 mM NTP was used. When the kinetic parameters K_m (mM) and V_m [μmol min^{-1} (mg of protein)$^{-1}$], from plots of $1/v$ vs. $1/[NTP]$, were determined, the NTP concentration from 0.1 to 2.0 mM was varied.

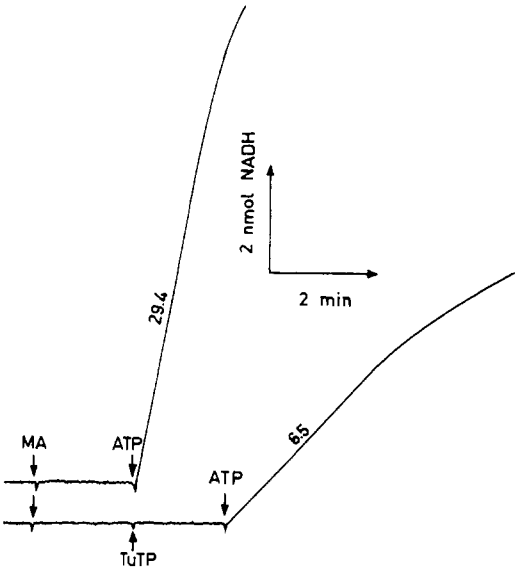

FIGURE 5: Effect of TuTP on energy-requiring reduction of NAD$^+$ by succinate. The experimental conditions were described under Materials and Methods. Protein (0.17 mg) of beef heart MA particles, 0.22 μmol of ATP, and 0.4 μmol of TuTP were used. The number beside the traces indicates the rate of NADH formation expressed as nmol [min^{-1} (mg of protein)$^{-1}$].

linear over a large interval of nucleotide concentrations when dATP, TuTP, or GTP was used as substrate, whereas the kinetic pattern with ATP as substrate showed the apparent negative cooperativity described by Schuster et al. (1975). It is clear from these data that the specificity of interaction of F_1 with ATP cannot be described solely by K_m and V_m values.

TuTP had no effect on the transhydrogenase reaction, while at the same concentrations, the effects of dATP and GTP were 57% and 27%, respectively, that of ATP. TuTP (0.6 mM) inhibits by 70% the transhydrogenase reaction driven by an identical concentration of ATP. TuTP had no effect upon the NAD$^+$ reduction by succinate but was a strong inhibitor of the ATP-stimulated reaction (Table IV and Figure 5). The measurement of the rate of NAD$^+$ reduction at different ATP concentrations (between 0.08 and 0.6 mM) without and with TuTP (between 0.05 and 0.4 mM) showed a typical compe-

Table V: Induction of Respiratory Control (RC) in MAU Particles by Nucleotide-Depleted F_1 and Different Nucleoside Triphosphates[a]

system	NADH oxidase activity [nmol min^{-1} (mg of protein)$^{-1}$] −FCCP	+FCCP	RC ratio
MA particles	122	324	2.65
MAU particles	272	258	0.95
MAU + F_1	252	278	1.10
MAU + ATP	277	293	1.06
MAU + F_1 + ATP	239	433	1.81
MAU + F_1 + TuTP	206	421	2.04
MAU + F_1 + dATP	274	374	1.37
MAU + F_1 + GTP	245	284	1.16

[a] A 0.02-mL sample of nucleotide-depleted F_1 in 50% glycerol corresponding to 0.1 mg of protein was incubated for 30 min at room temperature in a medium containing, at 0.2 mL, 100 mM Tris-sulfate (pH 8), 1 mM EDTA, and 2.5 mM NTP. Thereafter, 1.3 mg of MAU particles, 1 mg of bovine serum albumin and 1 μmol of Mg_2SO_4 in a total volume of 0.05 mL were added and incubated for another 30 min at +2 °C. At the end of this time, aliquots were withdrawn for measurement of NADH-oxidase activity, exactly as described by Tuena de Gómez-Puyou (1979). The ATPase activity of the particles using the regenerative assay system (1 mM ATP + 1 μM FCCP) was equal to 1.06 μmol min^{-1} (mg of protein)$^{-1}$ (MA particles) and 0.23 μmol min^{-1} (mg of protein)$^{-1}$ (MAU particles), respectively. The ATP + ADP content of the particles was 1.29 nmol (mg of protein)$^{-1}$ (MA particles) and 0.36 nmol (mg of protein)$^{-1}$ (MAU particles), respectively.

titive inhibition pattern (K_i = 0.045 mM). From the double-reciprocal plots, one obtained K_m^{ATP} equal to 0.16 mM, close to the K_m values for ATP hydrolysis with either soluble or membrane-bound F_1.

Urea treatment of the MgATP particles leads to important modifications in the enzymatic properties of the particles as a result of the removal of F_1 (Racker & Horstman, 1967; Pedersen & Hullihen, 1978; Tuena de Gómez-Puyou et al., 1979). The reconstitution of functionally active submitochondrial particles specifically requires ATP, with the other analogues excepting AMP-P(NH)P being inactive (Pedersen & Hullihen, 1978). As shown in Table V, TuTP resembles ATP, being capable of inducing respiratory control in MAU particles preincubated with F_1. Although the mechanism(s) through which ATP or TuTP improved the respiratory control ratios induced by F_1 is (are) not known, this could be used as a test for the reconstitution of active phosphorylating submitochondrial particles. As expected, F_1 alone or ATP alone was not able to induce a significant respiratory ratio in MAU particles; on the other hand, dATP, GTP, and several other ATP analogues were less effective than ATP and TuTP.

Discussion

Tubercidin was shown to exhibit severe cytotoxic effects on a variety of mammalian cell strains in vitro and significant antitumor activity in vivo (Owen & Smith, 1964; Saneyoshi et al., 1965; Bisel et al., 1970; Grage et al., 1970). The analogue was cytotoxic in all phases of the cell cycle (Bhuyan et al., 1972). Nitrobenzylthioinosine, a potent inhibitor of nucleoside transport, had a protecting effect on cells proliferating in culture against inhibitory concentrations of tubercidin (Patterson et al., 1979).

Our data demonstrate that the key role in the rapid phosphorylation of tubercidin in rat liver to TuTP, through the sequence of reactions involving adenosine kinase, adenylate kinase, and NDP kinase, belongs to adenylate kinase which has a high degree of specificity for AMP (Noda, 1973; Criss et al., 1970). On the basis of the kinetic parameters of the partially purified mitochondrial adenylate kinase (K_m^{AMP} = 0.067 mM and V_m^{AMP} = 50.4 μmol min^{-1} (mg of protein)$^{-1}$; K_m^{TuMP} = 1.42 mM and V_m^{TuMP} = 45.4 μmol min^{-1} (mg of protein)$^{-1}$ and of its high content in rat liver (135 units/g, with 10% being located in mitochondria) (Adelman et al., 1968), one can conclude that the liver mitochondria are able to phosphorylate in vivo TuMP at a rate practically equal to the rate of AMP phosphorylation. The mitochondrial NDP kinase, although representing only 4% of the liver enzyme (Jacobus & Evans, 1975), ensures a further fast phosphorylation of TuDP without the direct involvement of respiratory chain enzymes. In the case of heart mitochondria, there are at least two factors limiting the rate of TuMP phosphorylation to TuTP, namely, the considerably lower acceptor activity of adenylate kinase with TuMP as substrate and the different localization of NDP kinase, situated on the inner face of the inner mitochondrial membrane (Jacobus & Evans, 1975; Mantsch et al., 1975). These metabolic differences between liver and heart mitochondria could explain the relationship between the increase in the cellular concentration of TuTP (generally accompanied by a decrease in ATP) (Zimmerman et al., 1978) and the toxicologic effects of tubercidin. In fact, administration of tubercidin to rats causes a focal necrosis of hepatic parenchyma without significant alterations of the myocardium (Mihich et al., 1969). In pioneering studies on the cellular toxicity of tubercidin (Bloch et al., 1967), it was thought that the inhibition of growth of *Streptococcus faecalis* was primarily due to the interference of tubercidin with the utilization of glucose, mainly at the level of phosphofructokinase (Bloch, 1975). This cannot be the case in mammalian cells, since TuTP is one of the few nucleotides other than ATP which can support glycolysis of liver and heart homogenates. On the contrary, glucose formation from lactate by isolated perfused rat liver is rapidly and completely inhibited by 0.1 mM tubercidin (O. Bârzu et al., unpublished results).

One approach to understanding the mechanism of oxidative phosphorylation involves utilization of nucleotide analogues [for review, see Penefsky (1979)]. In this respect, the effects of TuDP and TuTP on mitochondrial oxidative phosphorylation deserve special attention. Although the role of the nucleotide binding sites in the mechanism of action of mitochondrial ATPase is still a subject of active investigation, it is agreed that at least one of the "tight" binding sites on the isolated F_1 may be a control site (Penefsky, 1979; DiPietro et al., 1980), whereas the catalytic activity involves only the "weaker" nucleotide binding site(s). However, in "coupled" submitochondrial particles, the "tight" nucleotide sites of F_1 are involved in both phosphorylation and hydrolysis (Harris et al., 1977). Several lines of evidence indicate that TuDP and TuTP preserve the ability of the natural nucleotides to interact with the "tight" binding sites of isolated or membrane-bound F_1: (a) Preincubation of F_1 with TuDP, similarly to ADP, inhibits the hydrolytic activity of the enzyme. (b) TuTP is highly efficient in restoring the "coupled" submitochondrial particles able to build up a H^+ gradient when MAU particles are incubated with F_1. This effect is independent of the hydrolysis of the analogue. Probably the interaction of TuTP [like ATP or AMP-P(NH)P] with one of the "tight" binding sites of F_1 induces a conformational change of the protein molecule which facilitates its binding to F_1-depleted membranes. (c) TuDP and TuTP are involved in "coupled" processes either as substrates (oxidative phosphorylation) or as inhibitors (reversal of electron flow). It was shown that the nucleotide specificity of the "coupled" processes is con-

siderably higher than that of the hydrolytic activity of F_1-ATPase and similar to that of the "tight" nucleotide binding sites (Garrett & Penefsky, 1975; Harris et al., 1978; Lascu et al., 1979). (d) Incubation of nucleotide-depleted F_1 with TuTP followed by washings with ammonium sulfate restores the original nucleotide content of the enzyme (2.7 mol of nucleotide/mol of F_1).

The low hydrolytic activity of F_1 in the presence of TuTP as substrate is more difficult to explain on the basis of our experimental data, particularly because the analogue displays properties similar to that of the natural compound in the hexokinase, creatine kinase, and phosphofructokinase reactions. We think that TuTP like other NTPs interacts with the catalytic site (the "weak" nucleotide binding site) of F_1 but unlike ATP, dATP, or GTP forms a relatively stable, less hydrolyzable complex. A possible structural factor responsible for this effect is the conformation of tubercidin nucleotides in aqueous solution. Whereas in the solid state tubercidin adopts the anti conformation like the natural adenine nucleotides (Abola & Sundaralingam, 1973), in aqueous solution its glycosyl bond is unusually flexible with a high accessibility to the syn conformation (Evans & Sarma, 1975), which is less favorable to hydrolysis by F_1. This hypothesis is supported by the enzymatic properties of 8-BrATP, an ATP analogue having the unusual syn conformation (Takenaka et al., 1978). 8-BrATP is weakly hydrolyzed by mitochondrial F_1 (Lascu et al., 1979) but can be quite successful in replacing the natural nucleotide when used with certain phosphotransferases such as NDP kinase (Nagel et al., 1976; Kezdi et al., 1976) or phosphoglycerate kinase (Lascu et al., 1979). Alternatively, one must postulate that F_1 does indeed contain separate sites for ATP hydrolysis and ATP synthesis with different specificities for nucleotides (Penefsky, 1974; Lee et al., 1977). This interpretation is supported by the differential inhibition of ATP formation or ATP hydrolysis by nucleotide analogues modified in the ribose ring or in the polyphosphate chain (Penefsky, 1974; Bârzu et al., 1979; Schäfer & Onur, 1979, 1980).

In conclusion, among ATP and ADP analogues, tubercidin nucleotides play a special role due to their minimal structural differences with respect to the natural compounds. In many reactions tubercidin nucleotides could "mimic" the behavior of adenine nucleotides, being good substrates for the phosphotransferases from mitochondria or cytosol. In several other systems these minimal structural modifications may lead to an almost total loss of enzymatic activity that could account for the observed inhibition of cellular metabolic processes.

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Functional Stability of *Torpedo* Acetylcholine Receptor. Effects of Protease Treatment†

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ABSTRACT: The effect of tryptic degradation on structural and functional properties of the membrane-bound acetylcholine receptor from *Torpedo californica* has been investigated. Under conditions of proteolysis which resulted in extensive degradation of receptor subunits, the membrane preparations retained their full capability of mediating agonist-induced cation flux as measured in rapid kinetic experiments. Low concentrations of trypsin also cleaved receptor dimers to monomers, and this effect was paralleled by degradation of the M_r 65 000 subunits which are known to contain sulfhydryl group(s) involved in receptor dimerization through an interchain disulfide bond(s). This conversion to monomers occurred at lower trypsin concentrations when the enzyme was added to the outside of the vesicles compared with the effects observed when the enzyme was present inside the vesicles. Similarly M_r 43 000 protein consistently found in preparations of the membrane-bound acetylcholine receptor, which can readily be removed without apparent effect on receptor function, displayed greater susceptibility to proteolysis when trypsin was added to the exterior medium rather than inside the vesicles. The results emphasize the full functionality of the monomeric form of the acetylcholine receptor comprised of four polypeptides.

The nicotinic acetylcholine receptor (AcChR)[1] from the electric organ of *Torpedo californica* is a pseudosymmetric complex of four homologous subunits of molecular weight 40 000, 50 000, 60 000, and 65 000 in the stoichiometry of 2:1:1:1 (Raftery et al., 1980). Following detergent extraction the receptor sediments on sucrose gradients as both a 9S

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[1] Abbreviations: AcChR, acetylcholine receptor; $NaDodSO_4$, sodium dodecyl sulfate; α-BuTx, α-bungarotoxin; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

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