(up to $10^{-4}M$ final concentration) in the medium. Vanadate concentrations ($1.5 \cdot 10^{-5}$ to $3 \cdot 10^{-5}M$), which cause little or no decrease in growth, failed to overcome inhibition by tungstate in N_2 fixation and in nitrate assimilation.

The data presented here point to a molybdenum system for N_2 fixation in *Azotobacter* in support of the reported stimulatory action of molybdenum observed by other investigators as indicated above. The results also suggest that a molybdenum enzyme, probably similar to that characterized in *Neurospora* and soy bean leaves, is involved in nitrate reduction in *Azotobacter*. It is of interest that tungstate has no effect (in final concentrations ranging from 10^{-6} to $10^{-3}M$) on the partially purified nitrate reductase from *Neurospora*. It is not unlikely that the *in vivo* inhibition by tungstate can be ascribed to its action in replacing molybdenum. In addition to its direct role in electron transport molybdenum apparently is necessary for the adaptive formation of nitrate reductase[4] during growth, and by extension, for the formation of the N_2-fixing enzyme system, presumably in the synthesis of the protein moiety(ies) of this system.

HAJIME TAKAHASHI*
ALVIN NASON

McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Md. (U.S.A.)

1 H. BORTELS, *Arch. Microbiol.*, 1 (1930) 333.
2 A. I. VIRTANEN AND N. RAUTANEN, in J. B. SUMNER AND K. MYRBÄCK, *The Enzymes*, Vol. 2, Academic Press Inc., New York, 1952, p. 1089.
3 D. J. D. NICHOLAS, A. NASON AND W. D. MCELROY *J. Biol. Chem.*, 207 (1954) 341.
4 D. J. D. NICHOLAS AND A. NASON, *J. Biol. Chem.*, 207 (1954) 353.
5 D. J. D. NICHOLAS AND A. NASON, *J. Biol. Chem.*, 211 (1954) 183.
6 D. J. D. NICHOLAS AND A. NASON, *Plant Physiol.*, 30 (1955) 135.
7 H. J. EVANS AND N. S. HALL, *Science*, 122 (1955) 922.
8 D. J. D. NICHOLAS AND A. NASON, *J. Bacteriol.*, 69 (1955) 580.
9 E. S. HIGGINS, D. A. RICHERT AND W. W. WESTERFELD, *Federation Proc.*, 15 (1956) 274.
10 E. S. HIGGINS, D. A. RICHERT AND W. W. WESTERFELD, *Proc. Soc. Exptl. Biol. Med.*, 92 (1956) 509.
11 C. K. HORNER, D. BURK, F. E. ALLISON AND M. S. SHERMAN, *J. Agr. Research*, 65 (1942) 173.
12 J. W. NEWTON, P. W. WILSON AND R. H. BURRIS, *J. Biol. Chem.*, 204 (1953) 445.
13 D. J. D. NICHOLAS, *Analyst*, 77 (1952) 629.
14 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.

Received October 20th, 1956

* Post-doctoral fellow of the McCollum-Pratt Institute on leave from Tokyo University, Tokyo, Japan.

The effect of thyroxine on the oxidative phosphorylation of tumour mitochondria*

Thyroxine has been shown to uncouple oxidation from the accompanying phosphorylations in the respiratory chain of liver mitochondria *in vitro*. In order to achieve this effect rather special conditions proved to be necessary. MARTIUS AND HESS[1], KLEMPERER[2] and HOCH AND LIPMANN[3] found that uncoupling took place only after preincubation of thyroxine with rat liver mitochondria. It was concluded that the hormone first had to pass the mitochondrial membrane before acting on the phosphorylating enzymes and that the rate of penetration was very slow with "intact" mitochondria. HOCH AND LIPMANN also studied mitochondria isolated from hamster liver. For thyroxine to act as an uncoupling agent, preincubation was not necessary with these mitochondria, which were considered relatively "leaky" in comparison with rat liver mitochondria. Recently TAPLEY, COOPER AND LEHNINGER[4] have shown that a 10 minute hypotonic treatment of rat liver mitochondria at 0° C is sufficient to result in a nearly complete uncoupling on subsequent incubation with thyroxine.

In the present experiments the ability of thyroxine to act as an uncoupler of tumour mitochondrial oxidative phosphorylation has been studied. The tumour mitochondria were *not* subjected to a special pretreatment intended to eliminate a possible structural barrier. This was considered unnecessary in view of (i) our earlier observations[5,6,7], which showed that the mitochondria from all the transplanted tumours studied exhibited a biochemical integrity that was less than that of liver mitochondria, and (ii) the possibility that this was due to, or at least accompanied by, a change in the mitochondrial structure and more especially in that of the membrane.

TABLE I

THE EFFECT OF THYROXINE ON THE OXIDATIVE PHOSPHORYLATION OF TUMOUR MITOCHONDRIA

Tumour mitochondria were isolated from homogenates in 0.25 *M* sucrose–0.001 *M* EDTA, pH 7.4, by centrifugation for 10 minutes at 5000 × *g* and washed twice by resuspension and centrifugation at 10,000 × *g*. 0.3 ml of the suspensions (approximating 0.5–0.7 mg N) were immediately added to Warburg flasks containing 100 μmoles KCl; 21 μmoles K phosphate buffer, pH 7.4; 10 μmoles ADP; 0.01 μmoles cytochrome *c*; 1.6 μmoles DPN; 35 μmoles $MgSO_4$; 16 μmoles NaF and 0.25 μmoles DL-thyroxine in the main compartment. After temperature equilibration (8 min), 12 μmoles DL-BHB, 40 μmoles glucose and 30 units (Kunitz-McDonald) hexokinase were tipped in. Total volume, 1.6 ml. The centre well contained 0.1 ml 3 *N* KOH. Incubated for 15 min at 27° C. O_2-uptake by standard Warburg technique. Phosphate determined by the method of FISKE AND SUBBAROW[12].

Mitochondria from	*Thyroxine*	$-\Delta P_i$ *μmoles*	$-\Delta O$ *μatoms*	*P:O*
T26473-hepatoma	—	2.8	1.0	2.8
	+	1.5	1.2	1.3
	—	4.5	1.9	2.4
	+	2.3	2.5	0.9
	—	3.3	1.1	3.0
	+	1.8	1.2	1.5
T5358-interstitial cell carcinoma testis	—	4.3	1.9	2.3
	+	1.5	1.2	1.3
T28012-hepatoma	—	7.7	3.7	2.1
	+	7.2	3.6	2.0
	—	5.5	4.7	1.2
	+	6.5	3.4	1.8
T5441-granulosa cell tumour ovary	—	3.6	2.4	1.5
	+	0.4	1.8	0.22

* The following abbreviations have been used: BHB for β-hydroxybutyrate, EDTA for ethylenediaminetetraacetate, DPN for diphosphopyridine nucleotide, ADP for adenosine diphosphate, P_i for inorganic phosphate, P:O for the ratio of the micromoles of inorganic phosphate esterified to the microatoms of oxygen utilized.

Tumour mitochondria that were the least intact from the biochemical point of view could not be studied since they did not oxidize readily[5,6,8]. Therefore, those tumour mitochondria that had been shown to be capable of oxidizing the D- but not the L-isomer of BHB were used. The particles were isolated in 0.25 *M* sucrose containing 0.001 *M* EDTA, pH 7.4, and immediately used for the measurement of oxygen and inorganic phosphate uptake in the absence and presence of thyroxine (DL-, $1.5 \cdot 10^{-4}$ *M*) and substrate. Addition of fluoride was necessary to effect net phosphate uptake in view of the adenosine triphosphatase activities of the mitochondria. DPN, glucose, hexokinase and Mg^{2+} were also present. The results of these experiments, illustrated in Table I, show that thyroxine markedly but not completely uncoupled the phosphorylations associated with the oxidation of DL-BHB by the mitochondria from the hepatoma T26473 and the testicular tumour T5358. In the last experiment listed in Table I, pyruvate together with a trace amount of L-malate served as substrate for the mitochondria from an ovarian tumour (T5441). The immediate uncoupling action of thyroxine is illustrated particularly well in this case since thyroxine was added from the side arm of the Warburg flasks after the period of temperature equilibration. Under the latter conditions using mitochondria from T26473 and DL-BHB a lowering of the P:O ratio from 2.7 to 1.2 was found.

However, an effect of thyroxine on oxidative phosphorylation was not a general property of tumour mitochondria, since the P:O ratios obtained with the mitochondria from another hepatoma (T28012) were not decreased. In a small number of experiments with mitochondria from this hepatoma and from some other tumours thyroxine has even been found to favour phosphorylation (*cf.* ref. [11]).

The uncoupling effect of thyroxine was reached with particles isolated in the presence of EDTA and incubated in the presence of Mg^{2+}, both of which compounds may counteract some of the changes induced by the hormone in liver mitochondria[9,10].

P:O ratios of mouse and rat liver mitochondrial controls were not affected by thyroxine.

In conclusion it may be stated that thyroxine has ready access to certain tumour mitochondria *in vitro*.

Department of Biochemistry,
Antoni van Leeuwenhoek-Huis, The Netherlands Cancer Institute,
Amsterdam (The Netherlands)

P. EMMELOT
P. J. BROMBACHER

1 C. MARTIUS AND B. HESS, *Biochem. Z.*, 191 (1955) 326.
2 H. G. KLEMPERER *Biochem. J.*, 60 (1955) 122.
3 F. L. HOCH AND F. LIPMANN, *Proc. Natl. Acad. Sci., U.S.*, 40 (1954) 909.
4 D. F. TAPLEY, C. COOPER AND A. L. LEHNINGER, *Biochim. Biophys. Acta*, 18 (1955) 597.
5 P. EMMELOT AND C. J. BOS, *Rec. trav. chim.*, 74 (1955) 1343.
6 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 19 (1956) 565.
7 P. EMMELOT, C. J. BOS AND P. J. BROMBACHER, *Brit. J. Cancer*, 10 (1956) 188.
8 P. EMMELOT AND C. J. BOS, *Enzymologia*, (in the press).
9 A. L. LEHNINGER, in O. H. GAEBLER, *Enzymes: Units of Biological Structure and Function*, Academic Press, New York, N.Y., 1956, p. 217.
10 J. H. PARK, B. P. MERIWETHER, C. R. PARK, S. H. MUDD AND F. LIPMANN, *Biochim. Biophys. Acta*, 22 (1956) 403.
11 C. MARTIUS 5. *Colloquium der Gesellschaft für physiologische Chemie, (Mosbach/Baden)*, Springer Verlag, Berlin, 1954, p. 143.
12 C. H. FISKE AND Y. SUBBAROW *J. Biol. Chem.*, 18 (1929) 629.

Received November 19th, 1956

The exclusive assay of the pro-activator of plasminogen by lysis of bovine fibrin clot

The pro-activator of plasminogen is a factor in blood plasma that forms a stoichiometric complex with streptokinase, which in turn acts catalytically in plasminogen activation[1].

A now obsolete method of "plasmin" assay depended on determining the rate at which standard clots of bovine fibrin were lysed in the presence of the plasmin preparation[2]. However, the discovery firstly that the bovine fibrinogen employed contained plasminogen[3] and secondly that the plasma activator was involved in the reaction[4], indicated that the lytic powers of the preparation might be determined by the content of pro-activator, plasmin or plasminogen. In fact, it was demonstrated[5] that under the conditions of assay then employed, the pro-activator predominated in determining potency.

It appeared that the lytic system might be modified to form a sensitive and rapid method of measuring pro-activator. In subjecting the assay to an elementary kinetic analysis, it has been assumed that the interaction of streptokinase and pro-activator is so rapid that it does not determine the rate of lysis[6,1].

Let

a = concentration of pro-activator in the system
p = concentration of plasmin formed by time t
F = the quantity of fibrin that must be digested in order that the clot may lyse
D = extent of fibrin digestion at time t
T = clot life.

If now streptokinase and plasminogen are present in excess

$$\mathrm{d}p/\mathrm{d}t = k_1 a$$

and

$$p = k_1 a t$$

Hence the rate of proteolysis

$$\mathrm{d}D/\mathrm{d}t = k_2 p = k_1 k_2 a t$$

and

$$D = (k_1 k_2/2)\, a \cdot t^2$$

where k_1 and k_2 are rate constants.

At the time T, D will be equal to F, which is a constant,

$$\therefore a = k\, 1/T^2$$

where

$$k = 2F/k_1 k_2.$$

Hence

$$\log a = \log k + 2 \log 1/T$$