Functional State of Mouse Liver Mitochondria in Toxicoses Due to *Aspergillus flavus* and *Penicillium rubrum*

Two strains of fungi, one of *Aspergillus flavus* and one of *Penicillium rubrum*, were detected in our laboratory to be hepatotoxic to mice, when infected with the diet[1]. Studies on citric acid cycle enzymes in these livers showed large deviations from those of normal. As most of these enzymes are present in mitochondria, it is desirable to examine the functional state of mitochondria in the affected livers which might explain the variations stated above and may also indicate whether mitochondria are vulnerable to attack by the toxic principles. For this, certain specific mitochondrial functions, such as oxidative phosphorylation, ATPase activity, pyridine nucleotide linked substrate oxidation and fatty acid oxidation have been examined.

Materials and methods. The diets were prepared as described elsewhere[1] and fed to Swiss albino mice (weighing about 20 g) for 4 weeks. On the 28th day, they were killed by cervical dislocation, livers were removed and mitochondria isolated as described by BRACHET and MIRSKY[2] (0.25 *M* sucrose medium at pH 8.5 for ATPase and at pH 7.4 for all other reactions). Protein was estimated by the method of LOWRY et al.[3], phosphorus by that of FISKE and SUBBAROW[4]. The ATPase activity was determined[5,6] in the absence of any added factors, in the presence of 2,4-dinitrophenol (DNP), EDTA and DNP and Mg^{++}. All reaction systems were incubated at 30 °C for 20 min, deproteinized with trichloroacetic acid and the phosphorus estimated. Oxidative phosphorylation was measured as described by HUNTER[7]. Oxidation of 2-oxoglutarate was carried out at 30 °C in the presence and in the absence of DNP by manometric method[8]. Oxidation of octanate was also followed manometrically.

The results are presented in the Tables I, II and III.

Discussion. Intact liver mitochondria have little or no ATPase activity and only in the presence of certain uncoupling agents such as DNP, the enzyme exhibits activity[9]. Addition of Mg^{++} to intact mitochondria does not activate ATPase[10]. But structural damage to mitochondria, such as that caused by freezing and thawing or by deoxycholate, stimulate ATPase in the presence of Mg^{++} while no DNP is present in the system[11]. Table I shows that in normal and in *A. flavus* poisoned liver, the ATPase activity is enhanced on addition of DNP, but not in the presence of added Mg^{++}. This indicates that there is no structural damage of mitochondria in *A. flavus* poisoned liver. But in the *P. rubrum* poisoned tissue, ATPase is not stimulated either by Mg^{++} or by DNP in the presence of EDTA or in its absence, and this indicates impaired functioning of mitochondria. Addition of EDTA to the system suppresses ATPase in normal, *A. flavus* and *P. rubrum* poisoned liver mitochondria. This is in agreement with the view of SELWYN[12], who found that EDTA inhibits purified ATPase.

The impaired oxidative phosphorylation and ATPase activity in *P. rubrum* poisoned tissue suggest that the structural integrity of the mitochondria is lost. However, oxidations of 2-oxoglutarate and octanoate proceed at normal rates. In *A. flavus* poisoned liver, the mitochondria appear intact, as all specific functions studied are well retained. The derangements observed in the mitochondrial functions in *P. rubrum* poisoning may be the primary lesions or only secondary effects of the key lesions, effected by the direct action of the toxic principle in the infected diet. In aflatoxin poisoning of rat liver, nucleus had been reported to be the primary locus of attack[13].

Table I. Activity of ATPase

Mitochondria of	Activity of ATPase (μg Pi/mg protein of mitochondria)			
	no added factors	in presence of DNP (30 μmoles)	in presence of DNP and EDTA (2 μmoles)	in presence of Mg^{++} (3 μ*M* $MgCl_2$)
Normal liver	0.8 ± 0.12	16.2 ± 0.34	10.7 ± 0.20	2.1 ± 0.88
A. flavus affected liver	1.0 ± 0.14	16.7 ± 0.28	9.8 ± 0.47	2.8 ± 0.25
P. rubrum affected liver	0.6 ± 0.10	0.8 ± 0.15	0.2 ± 0.05	0.8 ± 0.12

3 ml of the reaction system contained 6 μmoles of ATP; 30 μmoles of *Tris* buffer (pH = 8.5); 325 μmoles or sucrose and 0.5 ml of the mitochondrial suspension.

Table II. Oxidative phosphorylation

Mitochondria of	μ atoms of O or P/μg of protein of mitochondria		
	μ atoms of P	μ atoms of O	P/O ratio
Normal liver	2.70 ± 0.20	1.40 ± 0.10	1.92
A flavus affected liver	3.15 ± 0.21	1.50 ± 0.20	2.10
P. rubrum affected liver	2.08 ± 0.16	2.60 ± 0.20	0.80

2.7 ml of the reaction system contained 0.4 ml of 0.1 *M* potassium phosphate buffer, 0.2 ml of 1% cytochrome C; 0.2 ml of 0.1 *M* $MgCl_2$; 0.2 ml of 0.1 *M* ATP; 0.3 ml of 0.1 *M* succinate and 0.5 ml of mitochondrial suspension in the main compartment, 0.2 ml of 0.3 *M* glucose and 0.5 ml of the hexokinase preparation in the side-arm and 0.2 ml of 20% KOH in the centre-well.

[1] K. MADHAVIKUTTY and E. R. B. SHANMUGASUNDARAM, Proc. Indian Acad. Sci. *168*, 261 (1968).
[2] J. BRACHET and A. E. MIRSKY, in *The Cell* (Academic Press, New York and London 1959), vol. 1, p. 254.
[3] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).
[4] C. H. FISKE and Y. SUBBAROW, J. biol. Chem. *66*, 375 (1925).
[5] W. W. KIELLEY and R. K. KIELLEY, J. biol. Chem. *200*, 213 (1952).
[6] H. A. LARDY and H. WELLMAN, J. biol. Chem. *201*, 357 (1953).
[7] F. E. HUNTER JR., in *Methods in Enzymology* (Ed. S. P. COLOWICK and N. O. KAPLAN; Academic Press, New York 1955), vol. 2, p. 610.
[8] W. W. UMBREIDT, R. H. BURRIS and J. F. STAUFFER, in *Manometric Methods of Analysis* (Burgess Publishing Co., Minneapolis 1959).
[9] W. W. KIELLEY and R. K. KIELLEY, J. biol. Chem. *191*, 485 (1951).
[10] E. C. SLATER, in *Intracellular Respiration – Phosphorylating and Non-Phosphorylating Oxidation Reactions* (PWN, Polish Scientific Publishers, Warsaw 1963).
[11] V. H. PARKER, Biochem. J. *69*, 306 (1958).
[12] M. J. SELWYN, Biochem. J. *105*, 279 (1967).
[13] J. I. CLIFFORD and K. R. REES, Biochem. J. *102*, 65 (1967).

Table III. Oxidation of 2-oxoglutarate

Time (min)	µl of oxygen consumed/mg of protein of mitochondria					
	Normal liver		*A. flavus* affected liver		*P. rubrum* affected liver	
	in absence of DNP	in presence of DNP ($3\times10^{-5}M$)	in absence of DNP	in presence of DNP	in absence of DNP	in presence of DNP
20	1.20 ± 0.12	3.42 ± 0.34	3.22 ± 0.42	2.84 ± 0.40	1.45 ± 0.15	4.33 ± 0.60
40	2.83 ± 0.41	6.25 ± 0.44	6.74 ± 0.38	6.56 ± 0.32	3.16 ± 0.18	8.67 ± 0.32
60	4.61 ± 0.36	8.17 ± 0.78	7.45 ± 0.35	5.82 ± 0.26	4.58 ± 0.53	9.12 ± 0.82
80	5.18 ± 0.43	8.88 ± 0.80	7.62 ± 0.26	5.68 ± 0.32	4.82 ± 0.41	10.23 ± 0.76

2.7 ml of the reaction system contained 30 µmoles of inorganic P; 10 µmoles of $MgCl_2$; 30 µmoles of 2-oxoglutarate and 830 µmoles of sucrose in the main compartment; 0.3 ml of the mitochondrial suspension in the side-arm and 0.2 ml of 20% KOH in the centre-well.

The present studies indicate that mitochondria are not affected even secondarily until the focal necrotic stage of the poisoning (by *A. flavus*) at which these studies have been performed.

Zusammenfassung. Es wurde die funktionelle Integrität der Mitochondrien nach Verunreinigung der Nahrung mit *A. flavus* und *P. rubrum* aus vergifteter Mäuseleber untersucht. Die Mitochondrienfunktion der mit *P. rubrum* vergifteten Leber ist in bezug auf ihre ATPase-Tätigkeit und oxydative Phosphorylierung gestört, während im mit *A. flavus* vergifteten Gewebe alle wichtigen Mitochondrienfunktionen unverändert blieben.

K. Madhavikutty and
E. R. B. Shanmugasundaram

University Biochemical Laboratory, Madras 25 (India), 25 July 1968.

Distribution of Norepinephrine Uptake Within Rabbit Aorta Between Adventitia and Media[1]

Evidence has been presented that exogenous norepinephrine (NE) is taken up and bound to non-specific extraneuronal storage sites in tissues containing postganglionic adrenergic nerve fibers from which it can be released by tyramine[2-5]. In the present study, we have examined the uptake of tritium-labelled NE (^{3}H-NE) by neuronal and extraneuronal sites in the isolated rabbit aorta taking advantage of the anatomical arrangement in this vessel of 2 distinct circular layers: the smooth muscle containing tunica media and the adrenergic neurone containing tunica adventitia.

Methods. The general method described in detail by Nedergaard et al.[6], was used. Chromatographically pure (±)-7-^{3}H-norepinephrine hydrochloride (^{3}H-NE) was obtained commercially[7]. Rabbit aortic rings were placed in a tissue bath filled with physiological salt solution maintained at 37 °C. After appropriate incubation periods, the rings were removed, partially digested by means of a toluene-soluble quaternary base[8,9], and the radioactivity determined with a liquid scintillation spectrometer[10]. In some experiments following incubation with ^{3}H-NE the adventitia was stripped from the media in a manner similar to that described by Pease and Paule[11]. The completeness of the removal was confirmed histologically[12].

Extracellular space of intact aorta was determined using (carboxyl-^{14}C)inulin[7] (25 γ/ml).

Results. Rabbit aortic rings in vitro accumulated ^{3}H-NE (10^{-8} and $10^{-6}M$) when they were incubated with the labelled amine for varying time periods lasting from 2–60 min (Figure). Part of the uptake is accounted for by extracellular space. The mean uptake of (carboxyl-^{14}C)inulin after 60 min by 6 aortic strips was 0.47 ± 0.02 (S.E.M.) ml/g.

The relationship between extracellular concentration of ^{3}H-NE and the distribution of the uptake of this amine by aorta into adventitia and media was determined (Table). At a low concentration of ^{3}H-NE ($10^{-8}M$), the major part of this amine was taken up by adventitia, while only a small portion was localized in the media. As the bath concentrations of ^{3}H-NE was raised from $10^{-8}M$ to either 10^{-6} or $10^{-4}M$, the percentage of the

[1] Supported by grants from the U.S. Public Health Service (No. HE-08359) and The American Medical Association Education and Research Foundation.

[2] W. Haefely, A. Hürlimann and H. Thoenen, Br. J. Pharmac. *21*, 27 (1963).

[3] U. Trendelenburg and R. I. Pfeffer, Naunyn-Schmiedebergs Arch. exp. Path. Pharmak. *248*, 39 (1964).

[4] O. A. Nedergaard and E. Westermann, Naunyn-Schmiedebergs Arch. exp. Path. Pharmak. *251*, 163 (1965).

[5] J. E. Fischer, T. J. Kopin and J. Axelrod, J. Pharmac. *147*, 181 (1965).

[6] O. A. Nedergaard, A. Vagne and J. A. Bevan, J. Pharmac. *163*, 136 (1968).

[7] New England Nuclear Corporation, Boston, Massachusetts.

[8] NCS™ Solubilizer, Nuclear-Chicago Corporation.

[9] D. L. Hansen and E. T. Bush, Analyt. Biochem. *18*, 320 (1967).

[10] Unilux™ II, Nuclear-Chicago Corporation.

[11] D. C. Pease and W. J. Paule, J. Ultrastruct. Res. *3*, 469 (1960).

[12] The authors express their gratitude to Dr. M. A. Verity, Department of Pathology, UCLA School of Medicine, for performing the histology.