5. ———, *Protides of the Biological Fluids,* H. Peeters, ed., Elsevier, Amsterdam, 1963, v10, p. 131.

6. Higgins, G. M., Anderson, R. M., *A.M.A. Arch. Path.,* 1931, v12, 186.

7. Winzler, R. J., *Methods of Biochemical Analysis,* D. Glick, ed., Interscience, New York, 1955, v2, p. 279.

8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.,* 1951, v193, 265.

9. Scheidegger, J. J., *Intern. Arch. Allergy,* 1955, v7, 103.

10. Neuhaus, O. W., Havez, R., Biserte, G., *Arch. Biochem. Biophys.,* 1964, v106, 159.

11. Uriel, J., in *Analyse Immuno-Electrophoretique,* P. Grabar and P. Burtin, ed., Masson et Cie, Paris, 1960, p. 33.

12. Connell, G. E., Smithies, O., *Biochem. J.,* 1959, v72, 115.

13. Crosby, W. H., Munn, J. I., Furth, F. W., *U. S. Armed Forces Med. J.,* 1954, v5, 693.

14. Neuhaus, O. W., Sogoian, V. P., *Nature,* 1961, v192, 558.

15. Betsuyaku, I., *J. Jap. Soc. Int. Med.,* 1962, v51, 119.

16. Darcy, D. A., *Immunology,* 1960, v3, 325.

17. ———, personal communications.

18. Goa, J., *Acta Chem. Scand.,* 1960, v14, 1790.

19. Biserte, G., Havez, R., Hayem-Levy, A., *Clin. Chim. Acta,* 1960, v5, 272.

20. Schultze, H. E., Heide, K., Haupt, H., *ibid.,* 1962, v7, 854.

21. Benjamin, D. C., Weimer, H. E., *J. Immunol.,* 1963, v91, 331.

22. Chandler, A. M., Neuhaus, O. W., *Am. J. Physiol.,* 1964, v206, 169.

23. Betsuyaku, T., Yachi, A., Fukuda, M., Anzai, T., Wada, T., Carpenter, C. M., PROC. SOC. EXP. BIOL. AND MED., 1964, v115, 267.

24. Schumacher, G., Schlumberger, H. D., *Deut. Med. Wochensch.,* 1963, v88, 645.

25. ———, *Klin. Wochensch.,* 1962, v40, 81.

26. Jayle, M. F., Moretti, J., Mouray, H., *Les Haptoglobins,* M. F. Jayle, ed., Masson et Cie, Paris, 1962, p. 41.

27. Schumacher, G., Schlumberger, H. D., *Klin. Wochensch.,* 1962, v40, 67.

28. Jayle, M. F., Moretti, J., *Prog. in Hematology,* 1962, v3, 342.

29. Nyman, M., *Scand. J. Clin. Lab. Invest.,* 1959, v11, Supp. 39.

30. Robert, L., Mombelloni, P., Crosti, P., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 499.

31. Robert, B., Robert, L., Jayle, M. F., *Experientia,* 1959, v15, 385.

32. Laurell, C. B., Grönvall, C., *Adv. Clin. Chem.,* H. Sobotka and C. P. Steward, ed., Academic Press, New York, 1962, v5, 135.

33. Murray, R. K., Connell, G. E., *Nature,* 1960, v186, 86.

34. Neuhaus, O. W., Balegno, H. F., Milauskas, A. T., *Am. J. Physiol.,* 1962, v202, 257.

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Mechanism of Aminonucleoside-Induced Nephrosis in the Rat. IV. Hepatic Mitochondrial Oxidative Phosphorylation.* (29548)

PAUL BARTLETT, HEIDI SCHAEFER AND JANET KEEGAN

Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Mich.

Demonstration of significant reduction in the disappearance of inorganic orthophosphate associated with the oxidation of several tricarboxylic acid cycle intermediates by mitochondria prepared from the kidneys of rats treated with the nephrotogenic aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine(1,2,3), has raised several questions concerning the mechanism of action of this agent. *A priori,* if this finding is indicative of a direct effect of aminonucleoside on some aspect of the enzymatic reaction sequence involved in mitochondrial oxidative phosphorylation, then induction of similar effects in other tissue mitochondria should be demonstrable. Reported herewith are studies of oxidative phosphorylation in rat liver mitochondria during the course of induction of aminonucleoside-disease and in normal rat liver mitochondria incubated in the presence of added aminonucleoside.

Methods. The procedures and methods em-

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TABLE I. Time-Course of Development of Elevation in Liver Mitochondrial Oxidative Phosphorylation Associated with Oxidation of Succinate During Induction of Aminonucleoside Disease in the Rat.*

Group	No. of rats	Days of treatment*	Inorganic phosphate, $\Delta\mu$M/mg N†	Oxygen uptake, μAtoms/mg N†	P/O
I	15	None	14.8 ± 1.8	8.1 ± 1.5	1.8
II	6	2	15.5 ± 3.1	8.0 ± 1.3	1.9
III	5	4	21.2 ± 6.2	9.2 ± 1.4	2.3
IV	6	6	22.2 ± 4.2	10.0 ± 1.0	2.2
V	7	8	22.2 ± 6.7	10.8 ± 2.5	2.1
VI	5	10	27.6 ± 4.3	13.3 ± 2.8	2.1
Significance of differences:‡					
	Group III - I		$P = .01$	$<.2 > .1$	
	IV - I		$P < .01$	$<.05 > .02$	
	V - I		$P < .01$	$< .01$	
	VI - I		$P << .01$	$<<< .01$	

* 5 μM aminonucleoside/100 g body wt/day.
† Avg values ± standard deviations.
‡ Difference statistically analyzed by methods of Fisher(5).

ployed were essentially the same as those previously described(2,3) in studies of effects of aminonucleoside on phosphorylation associated with the oxidation of the tricarboxylic acid cycle intermediates—succinate, α-ketoglutarate, fumarate and malate—in rat kidney mitochondria. Oxidation of β-hydroxybutyrate was determined by estimation of acetoacetate formation as described by Walker(4). Acetoacetate concentrations were calculated by difference from determinations of the oxidation product on both heated and unheated protein-free filtrates from unincubated reaction mixtures, from complete reaction mixtures incubated for 20 minutes at 30°C, and from unincubated reaction mixtures in which the substrate was omitted and an equal volume of 0.15 M KCl substituted.

Results and discussion. The effects of induction of aminonucleoside-nephrosis on rat liver mitochondrial oxidative phosphorylation are in striking contrast to those previously reported for kidney mitochondria(2,3). Almost equal and opposite effects are induced. Thus, in 4 experiments with mitochondria prepared from the livers of aminonucleoside-treated (1.5 mg aminonucleoside/100 g body weight/day for 10 days injected subcutaneously) and normal control rats, average values for the disappearance of inorganic orthophosphate, associated with the oxidation of succinate, were respectively 23.6 ± 1.3 and 17.4 ± 1.1 (expressed in terms of μM orthophosphate/mg mitochondrial nitrogen). Corresponding oxygen uptake values were respectively 10 ± 0.3 and 6.5 ± 0.5 (μAtoms oxygen/mg mitochondrial nitrogen). Differences were statistically significant at the P level of 0.01(5).

Table I summarizes data obtained in a time-course study of the development of significant alterations in the disappearance of orthophosphate, associated with the oxidation of succinate, in liver mitochondria of rats treated with aminonucleoside. Analysis of the significance of the differences between the average values for the various groups clearly indicates significant elevation in the disappearance of inorganic orthophosphate as early as the 4th day during the period of treatment of the animals with aminonucleoside, and initiation of significantly higher oxygen uptake in liver mitochondria of rats sacrificed on the 6th day of drug administration. Now, while on the one hand, an increase in the phosphorylating capability of the rat liver mitochondria might be ascribed to a metabolic readjustment compensating for the loss of such activity in kidney tissue (1,2), the possibility must also be considered that aminonucleoside acts directly on enzymatic processes involved in hepatic mitochondrial oxidative phosphorylation.

Results summarized in Table II do, in fact, appear to support this view since incubating normal rat liver mitochondria directly with aminonucleoside in concentrations as low as 5.2×10^{-3} M, stimulates oxidative phos-

TABLE II. *In vitro* Effects of Aminonucleoside on Hepatic Oxidative Phosphorylation.

Exp	Aminonucleoside, molar	Inorganic orthophosphate, $\Delta\mu$M/mg N	Oxygen uptake, $\Delta\mu$Atoms/mg N	P/O
	Normal rat liver mitochondria			
I	None	10.9	8.8	1.24
	1.3×10^{-2}	18.7	9.5	1.97
II	None	11.1	8.1	1.37
	2.6×10^{-3}	11.7	9.5	1.23
	5.2×10^{-3}	13.5	10.5	1.29
	13.0×10^{-3}	16.6	10.9	1.52
	Normal rat liver submitochondrial phosphorylating complexes			
I	None	10.4	12.7	.82
	5.2×10^{-3}	7.4	11.6	.64
II	None	8.7	9.4	.93
	13.0×10^{-3}	6.8	11.3	.60

TABLE III. Effect of Aminonucleoside-Disease on Hepatic Mitochondrial Phosphorylation Associated with Oxidation of α-Ketoglutarate, Malate, and Fumarate.

Source of liver mitochondria	Substrate	Inorganic phosphate, $\Delta\mu$M/mg N†	Oxygen uptake, $\Delta\mu$Atoms/mg N†	P/O
Normal rats (4)	α-KG	18.40 ± 2.45	5.55 ± .96	3.32
Treated rats* (3)	α-KG	20.50 ± 1.56	5.47 ± .22	3.74
Normal rats (4)	Fumarate	6.99 ± 1.37	2.85 ± .56	2.45
Treated rats* (3)	Fumarate	7.34 ± .86	3.98 ± .52	1.84
Normal rats (3)	Malate	7.18 ± 1.35	3.69 ± .93	1.95
Treated rats* (3)	Malate	8.96 ± .12	3.53 ± .58	2.54

* Treated rats injected subcutaneously with 1.5 mg aminonucleoside/100 g body wt per day for a period of 10 days.

† Avg values ± standard deviations for number of experiments indicated in parentheses.

phorylation. Clearly, however, this effect of aminonucleoside on hepatic mitochondrial oxidative phosphorylation is dependent upon maintenance of the intact mitochondrial structure, for incubating aminonucleoside directly with normal rat liver submitochondrial phosphorylating complexes, prepared as described by Abood and Alexander(6), impairs phosphorylation associated with the oxidation of succinate. Results of such experiments are summarized in the lower part of Table II. In this respect, it seems of interest that the response of both liver and kidney preparations to added aminonucleoside is the same.

Table III summarizes the effects of aminonucleoside-nephrosis on hepatic mitochondrial oxidative phosphorylation associated with the oxidation of 3 additional tricarboxylic acid cycle intermediates. While phosphorylation associated with the oxidation of each of these substrates was impaired in kidney mitochondria of rats treated with aminonucleoside(3), no significant alterations were induced in liver mitochondria.

Concerning fatty acid oxidation in the aminonucleoside-nephrotic rat, Careddu and Apollonio have reported(7) the oxidation of octanoate to be significantly increased in both liver and kidney tissue slices. Our laboratory study of phosphorylation associated with the oxidation of β-hydroxybutyrate has failed to reveal any significant alteration. Results of 4 different experiments with mitochondria prepared from normal and aminonucleoside-nephrotic rat liver, for example, yielded average values of 9.6 ± 2.0 and 11.5 ± 0.9 (expressed as μM P_i/mg mitochondrial nitrogen) respectively for the uptake of inorganic orthophosphate, and average values of 2.5 ± 0.2 and 2.8 ± 0.4 (expressed as μM acetoacetate/mg mitochondrial nitrogen) respectively for acetoacetate formation. Of interest also and perhaps pertinent in this respect, are the findings of Marsh and Drabkin(8) in nephrotoxic serum nephrosis. Rates of incorporation of acetate-1-C^{14} into both liver

and plasma total fatty acids were considerably greater in nephrotic rats than in normal rats. In experiments with rat liver slices from control rats and from nephrotoxic serum nephrotic rats, however, no difference in the labeling of total fatty acids was noted.

Consideration has also been given to the extent to which phosphorylation, measured in terms of the disappearance of inorganic orthophosphate, is affected by the reverse process, dephosphorylation of adenosine triphosphate (ATPase activity). In this respect the stimulating effects of induction of aminonucleoside disease on hepatic mitochondrial oxidative phosphorylation become even more striking, for Mg^{+2} + Ca^{+2}-activated ATPase activity is significantly higher in liver mitochondria of aminonucleoside-nephrotic rats than in similar preparations from untreated normal control rats. In 8 experiments, average values of 47.5 ± 12.7 and 36.6 ± 9.4 (μM orthophosphate liberated/mg mitochondrial nitrogen) respectively were obtained in 20-minute assays conducted at 30°C.

From the present studies on liver and those previously reported for kidney(1,2), it seems readily apparent that induction of aminonucleoside disease results in the development of almost equal and opposite effects on mitochondrial phosphorylation associated with the oxidation of succinate. Concerning the nature of these differences we can only speculate. Relevant, however, may be differences in the ATP requirement of normal and aminonucleoside-nephrotic rat liver and kidney tissue. In rats in which extensive albuminuria has been induced with nephrotoxic serum, for example, Drabkin and Marsh(9) estimate plasma albumin synthesis to be 3- to 4-fold greater than it is in normal control rats, and further, report(8) net synthesis of albumin to be significantly higher in nephrotic than in normal rat liver slices. Thus, in the experimentally nephrotic rat the ATP requirement of liver tissue engaged in the energy-requiring process of plasma albumin synthesis would be expected to be higher than in kidney tissue in which breakdown of albumin appears to be a major metabolic process(10). While these considerations form an attractive explanation for the differences observed in liver and kidney mitochondrial oxidative phosphorylation in rats exhibiting massive proteinuria, they do not account for the induction of significant alterations in oxidative phosphorylation prior to onset and development of proteinuria.

Summary. 1. Phosphorylation associated with the oxidation of succinate is significantly higher in aminonucleoside-nephrotic rat liver mitochondria than in normal rat liver mitochondria. This alteration is induced as early as the 4th day during a 10-day period of induction of the disease with aminonucleoside. 2. Phosphorylation associated with the oxidation of α-ketoglutarate, malate, fumarate, and β-hydroxybutyrate is not significantly altered in liver mitochondria of the aminonucleoside-nephrotic rat. 3. Phosphorylation associated with the oxidation of succinate is also increased in normal rat liver mitochondria incubated directly with aminonucleoside. In similar experiments conducted with rat liver submitochondrial phosphorylating complexes, however, aminonucleoside inhibits oxidative phosphorylation.

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1. Bartlett, P., Ford, L., *Fed. Proc.*, 1960, v19, 38.

2. Bartlett, P., Keegan, J., Schaefer, H., PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 96.

3. Bartlett, P., in *Angiotensin and experimental renal disease*, 14th Annual Conf. Nat. Kidney Disease Fdn., Jack Metcoff, ed., Little, Brown & Co., Boston, Mass., 1963, pp221-225.

4. Walker, P. G., *Biochem. J.*, 1954, v58, 699.

5. Fisher, R. A., *Statistical methods for research workers,* Oliver & Boyd, Ltd., Edinburgh, 1948.

6. Abood, L. G., Alexander, L., *J. Biol. Chem.*, 1957, v227, 717.

7. Careddu, P., Apollonio, T., *Boll. Soc. Ital. Biol. Sper.*, 1960, v36, 569.

8. Marsh, J. B., Drabkin, D. L., *J. Biol. Chem.*, 1958, v230, 1083.

9. Drabkin, D. L., Marsh, J. B., *ibid.*, 1955, v212, 623.

10. Katz, J., Bonorris, G., Sellers, A. L., *J. Lab. and Clin. Med.*, 1963, v62, 910.

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