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Mechanism of Aminonucleoside-Induced Nephrosis in the Rat. III. Kidney Mitochondrial Phosphorylation and Dephosphorylation Activity.* (27961)

PAUL BARTLETT, JANET KEEGAN, AND HEIDI SCHAEFER
Edsel B. Ford Inst. for Medical Research, Henry Ford Hospital, Detroit, Mich.

During speculation on the chemical and physical nature of metabolic alterations which might explain the onset and development of the nephrotic syndrome in the rat treated with aminonucleoside, 6-dimethylamino-9-(3′ - amino - 3′ - deoxy - β - D - ribofuranosyl)-purine, the possibility was considered that some of the nephrotogenic action of the aminonucleoside might be due to interference with kidney mitochondrial phosphorylation and/or dephosphorylation reactions.

Results of exploratory studies of phosphorylation associated with oxidation of succinate might be considered as indirectly supporting the view that ATP formation is inhibited since both oxygen uptake and disappearance of inorganic orthophosphate are significantly reduced in kidney mitochondria of aminonucleoside-nephrotic rats(1). Several other considerations, however, suggest that this effect may be secondary in origin. Fisher *et al.*(2-3), working with kidney slices of aminonucleoside-nephrotic rats, report not only a significant depletion of histochemically demonstrable succinic dehydrogenase and cytochrome oxidase in the proximal convoluted tubules and ascending limbs of Henle, but also an apparent direct relationship between degree of depletion of the tubular enzymes and degree of proteinuria. This finding, together with the failure of aminonucleoside added directly to normal rat kidney slices to alter oxygen consumption, led these investigators to conclude that the alterations in enzymatic activity might be due to the proteinuria of the disease.

In addition to considering this possible explanation for our findings, the possibility must be considered that the observed reduction in disappearance of inorganic phosphate is due to a leak of phosphate from the organic to the inorganic orthophosphate pool. Thus dephosphorylation of adenosine triphosphate (ATP) and glucose-6-phosphate (G-6-P), both of which are formed in the oxidative phosphorylation reaction, and/or adenosine diphosphate (ADP), added as a phosphate acceptor, would either singularly or in combination effect an apparent reduction in disappearance of orthophosphate.

Studies were undertaken to determine the time-course of development of the proteinuria of the rat treated with aminonucleoside in relation to induction of the indicated alterations in kidney mitochondria(1); to evaluate the possibility of interference of dephosphorylation of ATP, G-6-P, and ADP with

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phosphorylation measurements; and to determine the *in vitro* effects, if any, of aminonucleoside on normal rat kidney mitochondrial oxidative phosphorylation and adenosine triphosphatase (ATPase) activity. The results are reported here.

Methods. Animals. Four- to five-month-old Sprague-Dawley rats, fed Rockland rat checkers *ad libitum,* were used in all studies. Animals treated with aminonucleoside were injected subcutaneously with a 1.5% solution prepared in isotonic saline. Doses of 5 μmoles/100 g body weight were administered subcutaneously daily for a period of 10 days or less, as desired. Following the final injection, the rats were fasted for 24 hours and then sacrificed in parallel with normal untreated rats from the same age group. Kidneys were immediately removed and placed in ice-cold 0.25 M sucrose.

Mitochondria. Chilled kidneys were decapsulated and a wedge-shaped section excised to remove connecting vessels and most of the pelvis. Mitochondria were prepared immediately as described by Weinbach(4). The final mitochondrial pellet was suspended in ice-cold 0.25 M sucrose and used within 30 minutes after preparation. Nitrogen content of the mitochondrial suspensions was determined by the procedure of Hiller *et al.*(5).

Oxidative phosphorylation studies. The composition of the reaction mixture used in the oxidative phosphorylation studies was essentially the same as that described by Weinbach and Garbus(6). Oxygen uptake was determined by the conventional Warburg technic, employing a 10-minute temperature equilibration period at 30°C, followed by a 15-minute period during which manometric readings were made at 5-minute intervals. Oxygen uptake for the initial 10-minute equilibration period was estimated by extrapolation to zero time. Inorganic phosphate concentrations were determined by the Fiske-Subbarow procedure(7). Net disappearance of inorganic orthophosphate was calculated from inorganic phosphate determinations conducted on zero time controls, on complete reaction mixtures incubated for 25 minutes, and on similarly incubated mixtures in which the substrate had been omitted. Urinary protein excretion was determined by the biuret method of Hiller *et al.*(8). Significance of differences was statistically evaluated by the methods of Fisher(9).

Dephosphorylating activities. Measurements of dephosphorylation of ATP, ADP, and G-6-P were made under conditions closely resembling those employed in the phosphorylation experiments. Hexokinase, glucose, and cytochrome *c,* were omitted from the reaction mixture and replaced by an equivalent volume of 0.25 M sucrose.

Results. Table I summarizes results of time-course study of the development of proteinuria and alterations in the disappearance of inorganic phosphate and oxygen uptake associated with the oxidation of succinate by kidney mitochondria of rats treated with aminonucleoside. Confirming the preliminary findings(1), the latter 2 were both significantly reduced in kidney mitochondria of rats (Group VI) treated for a period of 10 days with aminonucleoside. Statistical analysis of the difference between the average values for disappearance of inorganic phosphate for Groups I and III clearly indicates that this significant alteration is being established in rats treated with aminonucleoside for periods even as short as 4 days. Values of P lying between 0.02 and 0.05 were obtained. While similarly significant differences were not observed as early in oxygen uptake data, analysis of the difference between the average values for Groups I and IV, resulting in P values of 0.02 and 0.05, clearly indicates the development of impairment of this component of the oxidative phosphorylation system as early as the 6th day of treatment with aminonucleoside. Examination of the urinary protein excretion data reveals values lying within, or even below, the normal excretion range during the initial 4-day period of treatment. Extension of the treatment period to 6 days, however, results in a marked increase in urinary protein excretion, followed by progressive enhancement to the massive proteinuria seen in the 10-day aminonucleoside-nephrotic rat.

Results of studies of *in vitro* effects of aminonucleoside on phosphorylation, associated with the oxidation of succinate by nor-

TABLE I. Time-Course Study of Development of Impairment in Kidney Mitochondrial Oxidative Phosphorylation During Induction of Aminonucleoside-Nephrosis in the Rat.

Group	No. of rats	Days of treatment*	Urine protein† mg/24 hr	Inorganic phosphate‡ $\Delta\mu$M/mg N	Oxygen uptake $\Delta\mu$atoms/mg N	P/O
I	26	None	5.3 (2.1- 8.5)	28.8 ± 3.2	18.1 ± 2.2	1.6
II	4	2	1.0 (1.0- 1.5)	29.5 ± 3.2	20.7 ± 3.8	1.3
III	6	4	2.8 (0.5- 7.5)	24.0 ± 2.4	17.6 ± 2.4	1.4
IV	10	6	22.2 (4.4-41.9)	23.0 ± 1.8	15.3 ± 1.0	1.5
V	6	8	98.0 (46-188)	22.0 ± 4.1	15.2 ± .2	1.5
VI	6	10	261.0 (114-338)	14.6 ± 3.0	10.6 ± 2.1	1.4

Significance of differences:

Group I - III	$P = .02$-$.05$	
I - IV	$P < .01$	$P = .02$-$.05$
I - V	$P < .01$	$P = .05$
I - VI	$P << .01$	$P << .01$

* 5 μM aminonucleoside/100 g body wt/day.

† Average values for terminal 24 hr urine collections. Values in parentheses indicate range of urine protein excretion.

‡ Avg values/mg mitochondrial nitrogen.

± values are standard deviations.

mal rat kidney mitochondria, are summarized in Table II. In 3 different experiments, aminonucleoside in concentrations of 0.002, 0.004, and 0.01 molar—providing molar ratios of aminonucleoside to adenosine diphosphate of 1:1, 2:1, and 5:1—were tested. No significant changes were induced. Results of 3 similar *in vitro* experiments, in which normal rat kidney submitochondrial phosphorylating preparations, prepared as described by Abood and Alexander(10), were employed, are summarized in Table III. In contrast to experiments with intact normal rat kidney mitochondria, disappearance of inorganic orthophosphate was markedly reduced in Exp. 3. Oxygen uptake was not significantly altered.

TABLE II. *In Vitro* Effects of Aminonucleoside on Normal Rat Kidney Mitochondrial Oxidative Phosphorylation.*

Exp	Aminonucleoside concentration molar	Inorganic phosphate $\Delta\mu$M/ mg N†	Oxygen uptake μAtoms/ mg N†	P/O
1	None	36.1	18.3	1.97
	.002	36.1	15.4	2.34
2	None	40.8	20.8	1.96
	.004	38.6	21.7	1.78
3	None	56.4	26.9	2.09
	.01	53.4	26.7	2.00

* Composition of reaction mixture: Same as described by Weinbach and Garbus(6) with aminonucleoside added as indicated.

† Values expressed on basis of mitochondrial nitrogen.

TABLE III. *In Vitro* Effects of Aminonucleoside on Normal Rat Kidney Submitochondrial Phosphorylating Complexes.*

Exp	Aminonucleoside concentration molar	Inorganic phosphate $\Delta\mu$M/ mg N†	Oxygen uptake μAtoms/ mg N†	P/O
1	None	38.9	31.0	1.25
	.004	31.1	35.0	.89
2	None	42.0	35.4	1.19
	.004	38.5	38.2	1.01
3	None	30.6	22.6	1.35
	.004	27.3	27.9	.98
	.01	16.3	20.6	.79

* Composition of reaction mixture: Same as described by Weinbach and Garbus(6) with aminonucleoside added as indicated.

† Values expressed on basis of mitochondrial nitrogen.

Results of experiments designed to test the possibility that the observed reduction in disappearance of inorganic phosphate, associated with the oxidation of succinate by kidney mitochondria of the aminonucleoside-nephrotic rat, might be due to aminonucleo-

TABLE IV. Effects of Aminonucleoside-Nephrosis on Dephosphorylating Activity of Kidney Mitochondria.

Substrate	Dephosphorylating activity* Normal rats	Nephrotic rats	P values
	μM P_i liberated/mg mitochondrial N in 25 min.		
ATP	4.51 ± 1.82	5.41 ± 1.42	.05-.10
ADP	.23 ± .05	2.35 ± 1.91	.5 -.6
G-6-P	1.61 ± .7	.15 ± .70	->.9

* Average values for 3 different experiments.
± values are standard deviations.

side-stimulation of dephosphorylation of phosphate esters, known to be present in the reaction mixture, are summarized in Table IV. Under conditions closely resembling those employed in the oxidative phosphorylation studies, there appears to be no significant difference in dephosphorylation of ATP, ADP, or G-6-P by kidney mitochondria of normal or aminonucleoside-nephrotic rats. Even under experimental conditions considered optimal for assay of kidney mitochondrial ATPase, no significant differences were observed between the potential ATPase activity (11) of kidney mitochondria prepared from normal and from 10-day aminonucleoside-nephrotic rats. Average values ± standard deviations for 8 different experiments with kidney mitochondria from normal and treated groups were respectively 53.1 ± 12.7 and 59.6 ± 13.8. Statistical analysis of the difference between these activities gave a P value greater than 0.3. Of considerable interest, however, is the observation of aminonucleoside-inhibition of normal rat kidney mitochondrial ATPase in *in vitro* experiments. Results of such studies (Table V), suggest maximal inhibition at molar ratios of aminonucleoside to ATP of 1. This finding has been confirmed in more sophisticated experiments in which aminonucleoside was incubated directly with partially purified kidney mitochondrial ATPase, prepared and assayed essentially as described by Kielley and Kielley(12). Results of such an experiment are summarized in Table VI. It seems of interest that maximal inhibition of ATPase activity was again attained with a molar ratio of aminonucleoside to ATP of 1.

TABLE V. *In Vitro* Effects of Aminonucleoside on Normal Rat Kidney Mitochondrial ATPase.*

Aminonucleoside concentration	Phosphate liberated	% inhibition
molar	μM/mg mitochondrial N/20 min	%
0	17.4	
$5. \times 10^{-4}$	18.3	
1.25×10^{-3}	14.6	16.1
2.5×10^{-3}	8.5	51.2
5.0×10^{-3}	9.4	46.0
1.25×10^{-2}	12.6	27.6

* Composition of reaction mixture: ATP, .003 M; $MgCl_2$, .003 M; $CaCl_2$, .004 M; Sucrose, .133 M; mitochondria from 25 mg tissue (wet weight); aminonucleoside, molarity indicated. Incubation time, 20 min. at 37°C.

TABLE VI. *In Vitro* Effects of Aminonucleoside on Partially Purified Normal Rat Kidney Mitochondrial ATPase.*

Aminonucleoside concentration	Phosphate liberated	% inhibition
molar	μM/mg mitochondrial N/20 min	%
0	41.6	
2.5×10^{-3}	32.2	22.6
$5. \times 10^{-3}$	27.2	34.6
$10. \times 10^{-3}$	25.8	38.0

* Composition of reaction mixture: ATP, 5×10^{-3} M; $MgCl_2$, 5×10^{-3} M; Tris Buffer, pH 7.5, .11 M; KCl, .083 M; mitochondrial enzyme prep., total N, .13 mg; aminonucleoside, molarity indicated. Incubation time, 20 min. at 30°C.

Discussion. The time-course study data summarized in Table I not only demonstrate unequivocally the relatively early development of a significant reduction in disappearance of inorganic orthophosphate, associated with oxidation of succinate, during the period of induction of aminonucleoside-nephrosis, but also indicate the initiation of this physiological event prior to the onset of the functional aberration of proteinuria. From the results of studies of dephosphorylation of the phosphate esters, ADP, ATP, and G-6-P, under conditions closely resembling those employed in the oxidative phosphorylation experiments, as well as from the study of kidney mitochondrial ATPase activity in normal and aminonucleoside-nephrotic rats, and *in vitro* effects of aminonucleoside on normal rat kidney mitochondrial phosphorylation and ATPase activity, it appears justified to conclude that the observed reduction in disappearance of inorganic orthophosphate, asso-

ciated with the oxidation of succinate, is indicative of inhibited phosphorylation. Whether this represents a primary or secondary effect of aminonucleoside on oxidative phosphorylation however is not clear, for when aminonucleoside is added directly to normal rat kidney mitochondria, no significant alteration is observed in the disappearance of inorganic orthophosphate. *A priori* the latter might be considered as due to differences in *in vivo* and *in vitro* permeability of the mitochondrial membrane, for when aminonucleoside is incubated directly with submitochondrial phosphorylating complexes (Table III), inhibition of phosphorylation associated with oxidation of succinate occurs.

Of considerable interest in connection with our findings are those of Hess(13), who reports mitochondrial swelling in the basal portions of the epithelial cells of the proximal convoluted tubules to be the earliest observed alteration, occurring on the 4th day of aminonucleoside administration prior to onset of marked proteinuria and tubular distension. These findings, together with considerable evidence suggesting a reciprocal relationship between mitochondrial swelling and oxidative phosphorylation, and the suggestion of Di-Sabato and Fonnesu(14) that phosphorylation associated with oxidation of succinate may be the main mechanism by which mitochondrial swelling is prevented, lend further credence to the view that aminonucleoside-induced impairment in kidney mitochondrial oxidative phosphorylation may be involved in the mechanism by which this unusual nucleoside triggers its nephrotogenic activity.

Suggestion that aminonucleoside may produce some of its metabolic effects through inhibition of reactions by which the energy of ATP is released, as well as through inhibition of reactions producing energy-rich ATP, is found in the observation of *in vitro* aminonucleoside-inhibition of kidney mitochondria ATPase. While the actual role of ATPase in kidney mitochondria is largely a matter of conjecture, it is of interest to note the recent report by Skou(15) of the preparation of a purified enzyme system from mammalian kidney which hydrolyzes ATP to ADP and inorganic orthophosphate, and requires Mg^{+2}, Na^{+}, and K^{+} for full activity. The striking similarity of this preparation to erythrocyte membrane ATPase, identified as a part of the system involved in the active transport of sodium outward and potassium inward across the red cell membrane, supports the belief that the enzyme system from kidney tissue may be similarly involved. The preponderance of mitochondria at membrane boundaries in the kidney(16-18)—at which concentration gradients of sodium and potassium are maintained–as well as the presence at these same sites of cellular membranes relatively rich in ATPase activity(18-19) is certainly suggestive of functional significance. If such proves to be the case, then the finding of aminonucleoside-inhibition of ATPase activity may prove highly significant in understanding the general physiological problem of sodium retention and potassium excretion in the nephrotic syndrome.

Summary. Results of time-course studies of the development of a significant reduction in disappearance of inorganic phosphate, associated with oxidation of succinate in kidney mitochondria of aminonucleoside-nephrotic rats, clearly indicate the establishment of this effect as early as the 4th day during the induction of the nephrotic syndrome, and prior to onset and development of massive proteinuria. Oxygen uptake was not similarly altered until the 6th day of aminonucleoside administration and occurred in parallel with onset of proteinuria. Under experimental conditions closely resembling those employed in the oxidative phosphorylation studies, no significant differences were noted in dephosphorylation of ADP, ATP, and G-6-P by kidney mitochondria of either normal or aminonucleoside-nephrotic rats. These findings, and the additional observation of aminonucleoside-inhibition of kidney mitochondrial ATPase activity, appear to justify the conclusion that induction of aminonucleoside-nephrosis in the rat results in impaired kidney mitochondrial phosphorylation. Failure of aminonucleoside, added *in vitro* to normal rat kidney mitochondria, to significantly alter phosphorylation suggests that this effect is of secondary origin. Demonstration of inhibited phosphorylation when aminonucleoside is

added *in vitro* to normal rat kidney submitochondrial phosphorylating complexes, however, suggests a primary effect. It is suggested that the mechanism of action of aminonucleoside may be bimodal, producing some of its metabolic effects either directly or indirectly through inhibition of reactions producing energy-rich ATP and some directly through inhibition of reactions by which the energy of ATP is released.

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Pancreatic Hypertrophy and Chick Growth Inhibition by Soybean Fractions Devoid of Trypsin Inhibitor.* (27962)

H. C. SAXENA, LEO S. JENSEN AND JAMES MCGINNIS
Department of Poultry Science, Washington State University, Pullman

Discovery of the presence of trypsin inhibitors in soybeans(1,2) and other legumes (3,4), and the ability of concentrates of these factors to inhibit growth in rats(5,6) and chicks(7), led to the assumption that the trypsin inhibitor was responsible for the inferior nutritive value of raw soybean meal. However, contradictory results were later obtained and the mechanism of growth inhibition by raw soybean remains obscure. Feeding a purified trypsin inhibitor was shown to have an innocuous effect on growth of chicks (8,9) as well as of rats(8,10). Furthermore, fractions of beans (*Phaseolus vulgaris*) that were devoid of anti-tryptic activity inhibited growth markedly(11). Trypsin inhibitor concentrates prepared from raw soybean meal depressed fat absorbability, caused pancreatic hypertrophy, although the levels used were only slightly growth depressing(12).

Results of a study designed to determine if trypsin inhibitor was the factor that depressed growth and caused pancreatic hypertrophy in the chick are presented here.

Experimental. Crossbred (New Hampshire × Delaware) chicks were used in the experiments and were maintained in electrically heated, thermostatically controlled battery brooders with raised wire screen floors located in a temperature controlled laboratory. Feed and water were supplied *ad libitum*.

A semi-purified type diet (Table I) was used, which contained all nutrients known to be required by the chick. The heated soybean meal was prepared from soybean brew

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