Oxidative Enzyme Content of Kidneys from Normal and Aminonucleoside-Nephrotic Rats.* (31904)

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Daily injections of the aminonucleoside of puromycin, 6-dimethyl-amino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine, into rats produces the nephrotic syndrome in a uniform, reproducible manner(1). Fisher *et al* examined kidney slices from aminonucleoside-nephrotic rats histochemically and found that the stains for succinic dehydrogenase and cytochrome oxidase were less intense than those obtained on normal rat kidney slices(2). They also noted a significant reduction in Qo_2 values of kidney slices from the nephrotic rats. Investigations of oxygen uptake and disappearance of inorganic phosphate, associated with the oxidation of several tricarboxylic acid cycle intermediates as substrate, indicate that the rate of oxygen uptake and adenosine triphosphate (ATP) production with mitochondria from nephrotic rats is lower than with mitochondria from normal animals(3,4). Oxygen uptake rates by normal and treated rat kidney mitochondria in metabolic states 3 and 4,† as studied by the use of an oxygen electrode, suggested that the lowered rate of oxygen uptake by nephrotic rat kidney mitochondria in the presence of succinate and phosphate acceptor might be caused by a lowered activity of enzymes in the respiratory chain(6). This report compares (a) the cytochrome *c* content of whole kidneys from normal and nephrotic rats, and (b) the cytochrome *c* oxidase activity of kidney mitochondria from these two groups of animals.

Methods. Cytochrome c *analysis.* Four-month-old Sprague-Dawley virgin female rats were made nephrotic by 10 daily subcutaneous injections of aminonucleoside, 15 μg/g body weight. The treated rats were paired with normal controls and all procedures were carried out in parallel on a treated and a normal rat. During the tenth day of the treatment period, animals were fasted, and urine samples were collected for protein analysis(7). The rats were then sacrificed, the kidneys excised, rinsed with cold 0.25 M sucrose, decapsulated, and the pelvis removed. The kidneys were then weighed, minced, and ground with sand in glass mortars with successive additions of water, 0.5 M H_2SO_4, and 2 M NH_4OH to extract the cytochrome *c*. This is essentially the extraction procedure described by Fujita *et al*(8), and utilized by Rosenthal and Drabkin(9). The volume of the resulting homogenate was measured, and an aliquot removed for protein analysis(10), from which protein nitrogen content of the kidneys was calculated, using the factor 0.16.

The remaining homogenate was centrifuged at 2600 × *g* for 15 minutes. The supernatant was decanted and mixed with an equal volume of 50% $(NH_4)_2SO_4$ and immersed in a water bath at 56°C for 15 minutes to precipitate hemoglobin and some other spectroscopically inert proteins. Cytochrome *c* was then precipitated from solution by a combination of trichloroacetic acid and $(NH_4)_2SO_4$(9). After standing overnight at 0°C, the precipitate was collected by centrifugation at 36,000 × *g* for 20 minutes, and dissolved in a small volume of 1 M NaOH. The resulting solution was diluted, filtered, and brought to a final 5.0 ml (0.2 M NaOH) solution.

The optical density of this solution of cytochrome *c* in the oxidized form was measured at 550 mμ using a Beckman DU spectrophotometer. Sodium dithionite was then added in order to reduce the cytochrome *c* and the increase in optical density determined. The mμmoles of cytochrome *c* in the kidneys were calculated from this increase, using the values 9.1×10^3 and 27.6×10^3 liters moles^{-1} cm^{-1} for the oxidized and reduced forms, respectively(11). (Good recovery was obtained by this method when a known amount of cytochrome *c* in the reduced form was

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† Metabolic states defined by Chance and Williams(5).

added to a tissue preparation.) For purposes of comparison, the cytochrome *c* contents of kidneys were expressed in mμmoles/mg kidney protein nitrogen.

Cytochrome c *oxidase.* Enzyme activity was assayed at 25°C by the spectrophotometric method of Smith(12), using reduced cytochrome *c*. Cytochrome *c* (Sigma Chemical Co., Type III, from horse heart) solution, 80-88 μM, was reduced with H_2 in 0.01 M phosphate buffer, pH 7.0, using 5% platinized-asbestos (J. T. Baker Chemical Co.) as catalyst.

Three-month-old Sprague-Dawley virgin female rats were made nephrotic as described above. Using the method of Weinbach(13), mitochondria were isolated in parallel from normal and nephrotic rat kidneys, and suspended in 0.25 M sucrose (1:5). The protein concentrations of the suspensions were determined by a biuret procedure(14), with a 15-minute incubation period at 38°C, and the mitochondrial suspensions from the normal and treated rat kidneys adjusted to approximately equal protein concentrations using 0.25 M sucrose. Aliquots of these suspensions were then further diluted to a final 1:50 suspension with 0.25 M sucrose. The remaining (approximately 1:5) suspensions were kept for later nitrogen analysis by a micro Kjeldahl procedure(15). Suspensions (1:50) were kept at 0°C until assayed, and the assay carried out quickly following final dilution.

Activity of the mitochondrial enzyme was determined by placing 2.4 ml 0.1 M phosphate buffer, pH 7.0, 0.5 ml reduced cytochrome *c* solution, and 0.1 ml (1:50) mitochondrial suspension in a 3.0 ml cuvette, and following the oxidation of the cytochrome *c* photometrically at 550 mμ. Readings were taken at 30-second intervals for 3 minutes against a reagent blank. The optical density of the completely oxidized cytochrome *c* was read 3 minutes after the addition of 0.05 ml of saturated $K_3Fe(CN)_6$ solution. Triplicate (or quadruplicate) assays were carried out with each mitochondrial preparation. First-order rate constants were then calculated(12). Since the rate constants are proportional to mitochondrial enzyme concentrations in this concentration range, the calculated rate constants, for purposes of comparison, were divided by the μg mitochondrial nitrogen present in 1.0 ml of the reaction mixture. The standard deviations for triplicate (or quadruplicate) determinations on the separate mitochondrial preparations averaged $\pm 3 \times 10^{-3}$ $sec^{-1}/\mu g$ N $\times$ ml^{-1}.

TABLE I. Cytochrome *c* Concentration of Normal and Nephrotic Rat Kidney.

No. pairs of rats	Cytochrome *c** Normal†	Nephrotic‡	P§
	mμmoles/mg protein N		
6	.35 ± .05	.18 ± .04	<.001

* Average value using 6 rats and standard deviation of the average.
† Average urine protein excretion for terminal day was 4 mg.
‡ Average urine protein excretion for terminal day was 290 mg.
§ Significance level of difference between concurrently-run paired variates obtained by the *t* test (16).

An experiment was performed to ascertain what effects, if any, aminonucleoside might exert upon cytochrome *c* oxidase activity *in vitro*. Mitochondria were isolated from the kidney of a normal, non-fasted rat and suspended (1:25) in 0.25 M sucrose. An aliquot of this suspension was mixed (1:1) with a 0.25 M sucrose solution containing 0.02 M glycylglycine and 2×10^{-3} M disodium ethylenediaminetetraacetic acid (EDTA) at pH 7.4. Another aliquot was mixed (1:1) with 0.25 M sucrose solution, containing 0.02 M glycylglycine, 2×10^{-3} M EDTA, and 10^{-3} M aminonucleoside at pH 7.4. After standing 0.5 hour at 0°C, the rate constants of the two (1:50) mitochondrial suspensions were determined.

Results and discussion. The average kidney cytochrome *c* content (per mg protein nitrogen) of 6 normal and 6 nephrotic rats are shown in Table I, and the average cytochrome *c* oxidase activities of mitochondria isolated from 5 normal and 5 nephrotic rat kidneys are shown in Table II.

These results indicate that compared to normal controls both the cytochrome *c* content of the kidneys and the cytochrome *c* oxidase activity of isolated mitochondria are significantly reduced in aminonucleoside nephrotic rats. This confirms the earlier sug-

TABLE II. Cytochrome *c* Oxidase Activity of Normal and Nephrotic Rat Kidney Mitochondria.

No. pairs of rats	Cytochrome *c* oxidase activity* Normal†	Nephrotic‡	P§
	($sec^{-1}/\mu g$ N × ml^{-1}) (× 10^3)		
5	4.9 ± 1.1	3.3 ± .3	<.02

* Average value using 5 rats and standard deviation of the average.

† Average urine protein excretion for terminal day was 3 mg.

‡ Average urine protein excretion for terminal day was 250 mg.

§ Significance level of difference between concurrently-run paired variates obtained by the *t* test (16).

gestion that there is lowered respiratory chain enzyme activity in the kidney of the nephrotic rat(6). The per cent reduction found in cytochrome *c* oxidase activity (33%) agrees with the average reduction in oxygen uptake in state 3 found previously for isolated aminonucleoside-nephrotic rat kidney mitochondria (33%)(6), and with the reduction in Qo_2 of nephrotic rat kidney slices (22%) found by Fisher *et al*(2). Hence the lowered activity of cytochrome *c* oxidase may be large enough to account for the lowered oxidation by aminonucleoside-nephrotic rat renal tissue. Kinoshita *et al* using the Warburg technique found slightly elevated cytochrome oxidase activities in kidney homogenates from aminonucleoside-nephrotic rats compared to normal controls(17). However, the direct oxidation of reduced cytochrome *c* by the isolated mitochondrial fraction probably more nearly represents the respiratory activity of the electron chain during oxidative phosphorylation. Jansky has recently pointed out that total cytochrome oxidase activity serves as an indicator of the maximal metabolic capacities of animals by virtue of the function of the enzyme as terminal oxygen acceptor. Measurements of cytochrome *c* oxidase activity can be employed for comparisons of maximal metabolism between different animals and also between different organs of the same animal (18). This criterion might also be used to estimate the maximal oxidative capacity of mitochondria from cells of the same organ in different states of health. In the case of mitochondria from kidneys of aminonucleoside-nephrotic rats, the maximal oxidative capacity appears to be substantially reduced from normal values.

Although aminonucleoside, unlike puromycin, is not a specific inhibitor of protein synthesis(19), a reason for lowered enzyme activity and concentration in aminonucleoside-nephrotic kidney is suggested by reports of an inhibitory effect of aminonucleoside upon ribonucleic acid (RNA) biosynthesis in spheroplasts of *E. coli* B(20), the L-929 strain of mouse fibroblast(21), HeLa cells (22), and mouse small intestine(23). Selective inhibition in the biosynthesis of certain species of RNA by aminonucleoside could possibly also occur in the kidney and lead to decreased biosynthesis of certain proteins. Decreased production of enzymes involved in oxidative phosphorylation, such as cytochrome *c* or cytochrome *c* oxidase, might be expected to entail metabolic consequences and, thereby, contribute to the final syndrome found in aminonucleoside-nephrosis.

The *in vitro* experiment was performed to determine whether or not aminonucleoside exerts a direct inhibitory effect on the enzyme cytochrome *c* oxidase of normal rat kidney mitochondria. No significant difference in rate constants was observed between mitochondria suspended in solutions containing 5×10^{-4} M aminonucleoside and mitochondria suspended in solutions containing no aminonucleoside. Hence, there is no evidence, on the basis of this experiment, to indicate that the lowered cytochrome *c* oxidase activity found in aminonucleoside-nephrotic rat kidney mitochondria is caused by a direct inhibitory action of aminonucleoside upon this enzyme.

Summary. Cytochrome *c* contents of aminonucleoside-nephrotic rat kidneys and cytochrome *c* oxidase activities of aminonucleoside-nephrotic rat kidney mitochondria are significantly reduced in comparison with normal control values.

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Digestion of Articular Cartilage by Papain *in vitro*.* (31905)

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Crude papain and its purified, crystalline protease have been shown to have marked chondrolytic properties following systemic(1, 2), intraosseous(3) and intra-articular injection(4) of various species of laboratory animals. Reported studies of the effect of papain on cartilage *in vitro* have been essentially qualitative and somewhat contradictory, due possibly to variations in the preparations of papain that were used. Allison *et al*(5) stated that the ear cartilage of an adult rabbit became soft and lost its metochromatic staining properties following its immersion in a solution of crude papain at 37°C for 22 hours. Slack(6) found that the digestion of rat costal cartilage by purified papain for 48 hours resulted in the release of most of the polysaccharides into the supernatant solution. Curtiss and Klein observed that 75% of the hexosamine of bovine articular cartilage was lost when cartilage slices were incubated in a solution of an unspecified type of papain at 37° for 10 days, but only minimal dissolution of cartilage was observed(7).

It has been recently found that multiple intra-articular injections of papain protease can produce bony ankylosis of a joint(4). This led to the present quantitative investigation of the digestion of articular cartilage by papain *in vitro* by measuring the rate of appearance of various degradation products of cartilage during its disintegration in a solution of papain.

Materials and methods. Articular cartilage was excised with a scalpel from the upper tibia and lower femur of a 2-year-old steer about 5 hours after it had been slaughtered. The cartilage slices were immediately diced into fragments about 2 mm on edge, weighed out in 200 mg portions and stored at −10°C in tightly capped vials.

Papain protease, twice crystallized, was

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