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EFFECTS OF THE POTENT NEPHROTOGENIC AMINONUCLEOSIDE OF PUROMYCIN ON MITOCHONDRIAL MECHANOCHEMISTRY*

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Abstract—Neither spontaneous swelling nor U-factor formation is significantly altered in kidney mitochondria of rats treated for a period of 4 days with the nephrotogenic aminonucleoside of puromycin. Administration of the drug for an additional 6-day period, however, results in significant increases in initial swelling rates, extent of swelling, and level of U-factor in kidney mitochondria. Incubation of either normal rat kidney or liver mitochondria with 10^{-4} M aminonucleoside results in enhancement of initial swelling rates, with the effect on liver mitochondria being considerably greater than that on kidney mitochondria. Aminonucleoside *in vitro* also enhances the initial rate of swelling induced in liver mitochondria with orthophosphate. Since little or no change occurs in U-factor formation during the initial period of orthophosphate-induced swelling, a mechanism of aminonucleoside enhancement of mitochondrial swelling that operates independently of U-factor formation may be assumed. Incubation of normal rat liver mitochondria in the presence of an equimolar concentration of adenine, aminonucleoside, or adenosine monophosphate results in a reduction in the time of onset of orthophosphate-induced swelling, this reduction being least with adenine and greatest with adenosine monophosphate.

SINCE the demonstration of a drug-induced nephrotic syndrome in the rat with the aminonucleoside of puromycin, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine,[1] several exciting observations revealing alterations in cellular bioenergetics at the mitochondrial level of subcellular organization have been reported. Thus, for example, as early as the fourth day during the course of induction of the experimental disease, and prior to the onset of characteristic alterations in electrolyte and fluid balance and of massive proteinuria, phosphorylation associated with the oxidation of succinate is significantly impaired[2] and adenosine triphosphatase (ATPase) activity of rat kidney mitochondria significantly increased.[3] Subsequently, respiratory control in rat kidney mitochondria is also impaired, with respiration in state 3 being significantly reduced.[4]

Strikingly similar in the order of development are relatively early changes in glomerular ultrastructure, followed by changes in proximal tubule cell mitochondria.[5] Thus as early as the fourth day during the course of induction of the nephrotic syndrome with aminonucleoside, Metcoff *et al.*[5] observed loss of the characteristic normal epithelial cell organization (i.e. foot processes) abutting the basement membrane, and by the seventh to eighth day, virtually complete fusion of the podocytes.

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Equally striking, and occurring about the seventh day, are changes in the proximal tubule cells. In parallel with disruption and loss of the normally oriented arrangement of mitochondria in the axis of transport of proximal tubule cell epithelium, a reduction in both size and number of mitochondria, as well as a marked alteration in mitochondrial membrane ultrastructure, was observed.

While from the foregoing observations one might conclude that significant impairment in oxidative phosphorylation and significant elevation in ATPase activity in kidney mitochondria of rats treated with aminonucleoside are initiating events in the sequential development of conformational changes in glomerular epithelial and tubular cell ultrastructure,[5-9] such a conclusion would still leave unsolved the formidable problem of the nature of the transducing system involved.

In this respect, consideration of mechanochemical parameters which might be involved in the transduction process has been profoundly influenced by results of studies of the phenomena of swelling and contraction in liver mitochondria,[10-12] and of the molecular organization of biological transducing systems.[13] Indeed, in the case of liver mitochondria, the discovery of the formation and disappearance of an endogenous uncoupling factor, i.e. U-factor, during swelling and contracting phenomena,[10-11] as well as the demonstrated capability of U-factor to stimulate ATPase activity and swelling, has provided an exciting common denominator linking mechanochemical changes in the mitochondrion with cellular respiration and bioenergetics.[12]

Virtually nothing is known concerning the nature of transducing systems in renal tissue, in which transduction of chemical energy into mechanical energy must be of considerable import. This, along with the possibility that stimulation of U-factor formation might be an initiating event resulting in impairment in oxidative phosphorylation and stimulation of ATPase activity in kidney mitochondria, has prompted study of swelling and U-factor formation in kidney mitochondria of normal and aminonucleoside-treated rats. Furthermore, similarities in chemical composition and morphological structure of cellular and mitochondrial membranes,[12, 14-16] and ease of isolation of mitochondria, suggest usefulness of the mitochondrial model in the study of the mode of action of agents known to induce changes in membrane transport and permeability.

METHODS

Preparation of mitochondria. Liver mitochondria, isolated essentially as described by Weinbach,[17] washed three times with ice-cold 0·25 M sucrose and finally suspended in 2 volumes of 0·25 M sucrose, buffered with 0·02 M Tris-HCl, pH 7·4, were employed in the assay of extracts of kidney mitochondrial U-factor. Since considerable improvement was noted in the recovery of U-factor (standard oleate) when 10^{-3} M ethylenediamine tetraacetic acid, pH 7·0, was added to the initial 0·25 M homogenizing sucrose solution, this procedure was adopted in preparing the kidney mitochondria. Washing and final suspension of the kidney mitochondria were accomplished as described for liver mitochondria.

Measurement of swelling. Mitochondrial swelling was determined optically at 520 mμ in 1-cm cells in the 25° thermostatted cell compartment of a Beckman DU Monochromator, coupled to a Gilford converter and Leeds and Northrup recorder.

The medium employed was the same as that used by Lehninger *et al.*[18] in an investigation of the spontaneous swelling rate of rat liver mitochondria. The suspending solution was 0·02 M in Tris-HCl and 0·125 M in KCl, pH 7·4. Mitochondrial suspensions were prepared to contain per ml the mitochondria from 7·3 to 10 mg wet tissue Optical densities were recorded at 0, 5, 10, 15, 30, 60, 120, and 180 min.

Measurement of U factor. The procedure employed for the extraction of U–factor and the assay of extracts was essentially the same as that described by Wojtczak and Lehninger.[11] Lipid-free bovine albumin (prepared by washing crystallized material twice with absolute ethanol and once with ethyl ether) was, however, added to the kidney suspensions prior to heat coagulation. Ethanolic extracts of the heat coagulum were evaporated to dryness under nitrogen and stored overnight at −15° prior to assay. Dry extracts were dissolved in absolute ethanol and assayed for U–factor at two levels of concentration. Results were expressed in terms of oleate equivalents producing the same extent of swelling. Protein concentrations were determined by the method of Gornall *et al.*[19]

Treatment of animals. Four- to five-month old Sprague–Dawley virgin female rats, fed Rockland rat checkers *ad libitum*, supplemented with fresh lettuce twice weekly, were used in all studies. Aminonucleoside was administered in isotonic saline solution, 5 μM being injected subcutaneously per 100 g body weight per day.

RESULTS

Results of studies of swelling and U-factor formation in kidney mitochondria of normal and aminonucleoside-treated rats are summarised in Table 1. Clearly, treat-

TABLE 1. SWELLING AND U-FACTOR FORMATION IN KIDNEY MITOCHONDRIA OF NORMAL AND AMINONUCLEOSIDE-TREATED RATS

Source of mitochondria	No. of experiments	Initial* swelling rate	Extent† of swelling (%)	U-factor concentration‡ Initial	Final	Formed
				(mμmoles/mg protein)		
I. Normal	13	31·4 ± 3·2	28·0 ± 2·9	0·9 ± 0·3	3·1 ± 1·0	2·2 ± 0·9
Treated:						
II. 4 days	6	31·3 ± 2·2	27·5 ± 2·3	0·8 ± 0·3	2·5 ± 0·3	1·7 ± 0·3
III. 10 days	6	40·8 ± 5·2	32·7 ± 3·2	1·5 ± 0·2	4·1 ± 0·7	2·7 ± 0·7
Significance of differences: P values from Fisher's *t* values[34]						
	Group III-I	<0·01	<0·01	<0·01	>0·02<0·05	

* Expressed as (changes in optical density at 520 mμ/min/mg mitochondrial protein) × 10^3.

† Per cent reduction in optical density at 520 mμ after 2-hr incubation at 25°.

‡ Initial, U-factor concentration in oleate equivalents prior to incubation. Final, concentration after 2-hr incubation.

ment of rats with aminonucleoside for 4 days—the period required for development of significant impairment in oxidative phosphorylation[2] and significant elevation in ATPase activity[3]—has no effect on the mechanochemical parameters investigated. After 10 days of aminonucleoside administration, however, the initial swelling rate, extent of swelling, and concentration of U-factor are all significantly increased.

As can readily be seen in the spontaneous swelling curves shown in Fig. 1 and the data summarized in Table 2, incubation of normal rat kidney or liver mitochondria in the presence of varying concentrations of the nephrotogenic aminonucleoside results in considerable enhancement of initial swelling rates. In the case of liver mitochondria, the initial swelling rate is more than doubled in the presence of 10^{-4} M

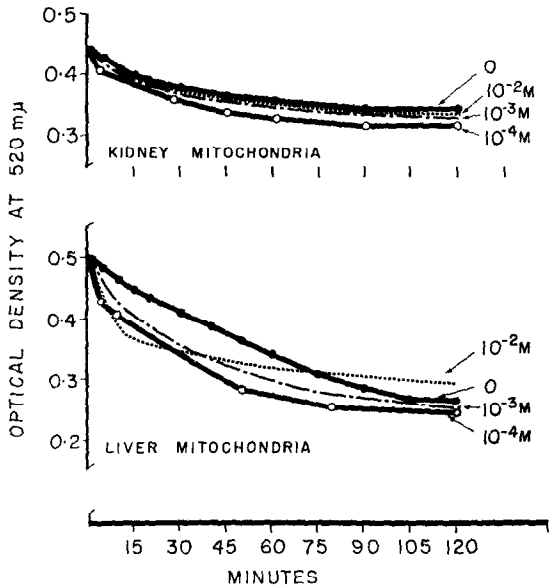

FIG. 1. Effects *in vitro* of aminonucleoside (10^{-2}—10^{-4} M) on the spontaneous swelling of normal rat kidney and liver mitochondria

aminonucleoside; while in the case of kidney mitochondria, the initial swelling rate is increased only 54 per cent. Pertinent to and perhaps related to this finding is a striking difference in the extent of swelling of normal rat kidney and liver mitochondria. Expressed in terms of percentage reduction in optical density of equivalent

TABLE 2. EFFECTS *IN VITRO* OF AMINONUCLEOSIDE ON SWELLING OF NORMAL RAT KIDNEY AND LIVER MITOCHONDRIA

Experiment	Initial swelling rate*		Extent of swelling†	
	Liver (%)	Kidney (%)	Liver (%)	Kidney (%)
Normal	8·3	6·7	50	21·4
Normal + 10^{-4} M aminonucleoside	17·4	10·3	53	26·4

* Per cent reduction in optical density at 520 mμ in initial 10-min period.
† Per cent reduction in optical density at 520 mμ in 2-hr incubation at 25°.

mitochondrial suspensions incubated for 2 hr at 25°, the extent of swelling of liver mitochondria is also twice that of kidney. In comparison with the marked enhancing effects *in vitro* of aminonucleoside on initial swelling rates of normal rat kidney and liver mitochondria, the extent of swelling is only slightly increased. This can be

clearly seen in the relative positions of the spontaneous swelling curves in Fig. 1 and the data summarized in Table 2. In this respect it also seems of interest to note that the effect is less evident at concentrations of aminonucleoside ranging from 10^{-3} to 10^{-2} than at the relatively low concentration of 10^{-4} M. In the case of liver mitochondria incubated in the presence of 10^{-2} M aminonucleoside, there is in fact a sharp break in the swelling curve at the 10-min mark, terminating in an actual reduction in extent of swelling.

Seeking further insight into the nature of the stimulating effects of the nephrotogenic aminonucleoside on mitochondrial swelling, we also conducted experiments with normal rat liver mitochondria incubated in the presence of aminonucleoside and a variety of agents known to inhibit swelling. If ATP, bovine serum albumin (BSA), or EDTA is added at the beginning of the incubation period, swelling is completely inhibited.

Now, while the primary event in spontaneous swelling may be U–factor formation, no significant increase in mitochondrial U–factor concentration is observed in the initial phase of rapid swelling induced by orthophosphate, nor is swelling inhibited by serum albumin capable of trapping U–factor.[11] For this reason, study of the effects of aminonucleoside on orthophosphate-induced swelling of liver mitochondria was also of interest. From the results of such studies, presented in Fig. 2, the en-

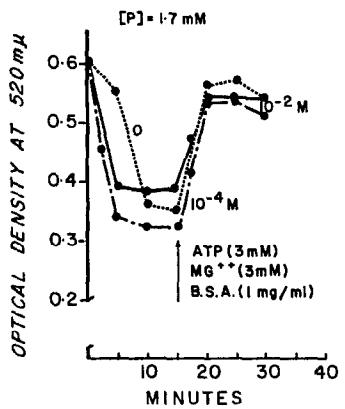

FIG. 2. Effects *in vitro* of aminonucleoside (10^{-2}—10^{-4} M) on the orthophosphate-induced swelling and ATP-induced contraction of normal rat liver mitochondria.

hancing effects of aminonucleoside on the initial swelling rate are clearly evident; and, as previously noted in the spontaneous swelling experiments, the presence of 10^{-2} M aminonucleoside increases the initial swelling rate and reduces the extent of swelling. The reversal of swelling and contraction of mitochondria brought about by the combined addition of ATP, Mg^{+2}, and BSA (as gauged by increase in optical density at 520 mμ) is unaffected by aminonucleoside.

Effects of equimolar concentrations of adenine, aminonucleoside, and adenosine monophosphate (AMP) on orthophosphate-induced swelling of liver mitochondria

are shown in Fig. 3. While the extent of swelling appears to be the same in all three cases, the time of onset of swelling seems to be progressively reduced as the structure of the added compound approaches that of the nucleotide. That the action on swelling is non specific is fairly obvious.

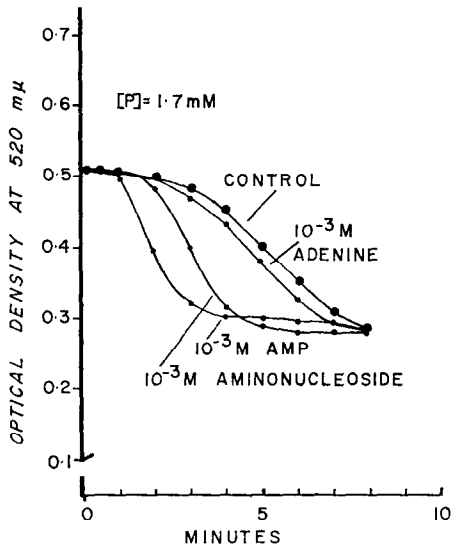

FIG. 3. Effects *in vitro* of adenine, aminonucleoside, and adenosine — 5′-phosphate on orthophosphate-induced swelling of normal rat liver mitochondria.

DISCUSSION

While at the start of this investigation the idea was entertained that a primary event in the induction of the aminonucleoside disease might be stimulation of U–factor formation resulting in uncoupling of oxidative phosphorylation and stimulation of ATPase activity, this can no longer be considered a tenable position. Celarly, development of the significant increase in U–factor occurs after rather than before or coincident with the significant impairment in oxidative phosphorylation[2] and significant elevation in ATPase activity.[3] The relatively late development of significant changes in mitochondrial swelling[20] and U–factor formation, however, correlates remarkably well with changes in mitochondrial ultrastructure, as seen in the proximal tubule cells with the aid of electron microscopy.[5, 9] These changes, therefore, may provide a mechanochemical basis for the focal swelling between the intramitochondrial membrane or cristae.[6]

Completely unexplained, however, are the relatively early changes seen in the normally well-defined foot processes adjoining the glomerular basement membrane.[5] Although results of the electron microscopy studies by Metcoff *et al.*[5] indicate the appearance of such alterations as early as the fourth day during the induction of the nephrotic syndrome in the rat with aminonucleoside, these clearly cannot be related to the mechanochemical parameters of increased U–factor formation and swelling rate of the kidney mitochondria.

Now whether swelling of kidney or liver mitochondria is permitted to occur spontaneously or is orthophosphate-induced makes no apparent difference in the

effects of aminonucleoside thereon *in vitro*, so far as can be detected by the procedures employed in this study. This in itself is an interesting finding, since in spontaneous swelling, U–factor levels rise synchronously with the rate of swelling,[11] while in the case of orthophosphate swelling, little or no change occurs in this factor.[11] Thus, even though the mechanism of induction of spontaneous and of orthophosphate-induced swelling differs in this respect, the site of interaction of aminonucleoside with the mitochondrion and the mechanism by which the nephrotogenic aminonucleoside enhances the initial swelling rate *in vitro* must be essentially the same in the two cases. While we can only speculate concerning the nature of chemical receptors in such interaction and the effect produced, observations of effects both *in vivo* and *in vitro* of aminonucleoside on ATPase activity of kidney mitochondria, reported in other communications from our laboratory,[2, 3] are strongly suggestive of the system involved. Conceivably, while such changes may not be detectable optically either by measurement of 520 mμ absorbance or by use of electron microscopy, they could lead to significantly altered membrane permeability and/or transport.

Indeed, preliminary results of a study of effects *in vitro* of aminonucleoside on myosin- and actomyosin-like ATPase preparations, prepared from kidney cortex by the procedures described by Perry,[21] provide an exciting lead for further study. In the presence of activating Ca^{+2} and $7{\cdot}5 \times 10^{-3}$ M aminonucleoside, the myosin-like activity was inhibited 75 per cent, and the actomyosin-like ATPase 65%,[22]

While in the case of muscle myofibril, myosin and actin are well-documented protein-transducing molecules, it is clearly evident that further study is required before a similar mechanoprotein concept can be invoked to explain swelling and contraction phenomena in mitochondria. Thus, even though KCl extraction of liver mitochondria yields protein-containing fractions having ATPase activity and some of the physical properties of muscle actomyosin, myosin, and actin,[23–25] relatively recent observations of Vignais *et al.*[26, 27] on such fractions seem to exclude the so-called contractile proteins from specific contraction supporting activity. Particularly pertinent in this respect are demonstrations of contraction-supporting activity in other mitochondrial protein fractions, of heat stability of such activity in all preparations, including that of Ohnishi and Ohnishi,[23, 24] and of a specific lipid requirement (protein-bound phosphatidyl inositol) for contractile activity.[27]

Aside from the possible involvement of contractile or mechanoproteins in the mechanism of mitochondrial swelling or other ultrastructural changes in glomerular epithelial or tubular cells, in the final analysis one must also consider mechanisms ascribable either to osmotic effects arising from altered active ion transport,[28–31] and/or to conformational changes in cellular morphology arising as a result of alterations in energy-linked intermediates generated by oxidative phosphorylation.[32,33] Certainly any one of these, any combination thereof, or all of them in concurrent operation may contribute significantly to maintenance of normal cellular integrity. It is to be hoped, from separate studies of these parameters, that significant information will be derived, permitting composite evaluation and better understanding of the pathogenesis of the disease process induced in the rat by the nephrotogenic aminonucleoside.

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