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Dose-Response Relationship in Aminonucleoside Nephrosis.* (28089)

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A possible mode of action of the aminonucleoside† (PA) of the antibiotic puromycin in inducing the nephrotic syndrome in rats is by blocking nucleic acid synthesis through enzyme inhibition(1-3). To test this hypothesis we have administered decreasing doses of PA subcutaneously at weekly or biweekly intervals instead of the usual daily dose of 1.5 mg/100 g. It was reasoned that the appearance of proteinuria under these circumstances would constitute evidence that *direct* enzyme inhibition was not involved, since at these low doses sufficient blood levels to block a postulated enzyme system would likely not be maintained, especially over such a protracted period. By varying the dose schedules in this manner, by administering PA into the circulating blood and by utilizing "stopped" schedules (1-4 consecutive daily doses), we found, unexpectedly, that the development of proteinuria depended not only on the total cumulative dose administered but also on reaching a certain threshold dose.

Methods. Female albino rats of the Holtzman strain weighing 200 ± 20 g were divided into groups of 3-6 rats each. Doses of PA ranging from 1-3 mg were administered subcutaneously, intravenously, or into the heart or aorta according to the schedule shown in Table I. For intravenous and intracardiac injections the rats were lightly anesthetized with ether. For intra-aortic administration, the drug was injected just above the renal arteries through a 26-gauge needle while the aorta at its bifurcation was temporarily obstructed by finger compression. The drug was prepared as a 0.50% solution in distilled water or isotonic saline and the actual volume injected was always less than 1 ml. Urine was collected daily or weekly and 24-hour protein concentrations were determined by the method of Shevsky and Stafford as modified by McKay(4).

TABLE I. PA Dosage Schedule.

Group	No. rats	Dose/rat (mg)	Route of admin	Interval
A	3	3	Subcutaneous	Weekly
B	3	3	"	Biweekly
C	3	2	"	"
D	4	1	"	"
E	6	3	"	Daily × 16
F	8	20	Intravenous	Single dose
G	4	3	"	Daily × 6
H	4	5	Intra-aortic	Single dose
I	4	5	Intracardiac	" "
J	12	3	Subcutaneous	Daily × 13
K	6	3	"	Single dose, repeated at 4 weeks
L	6	3	"	Daily × 2
M	6	3	"	" × 3
N	6	3	"	" × 4

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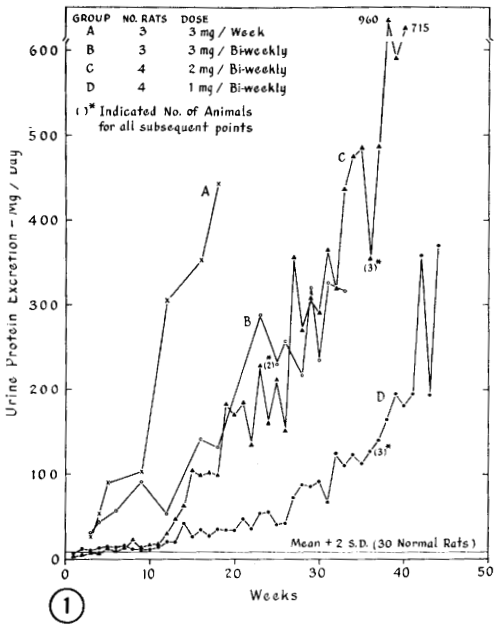
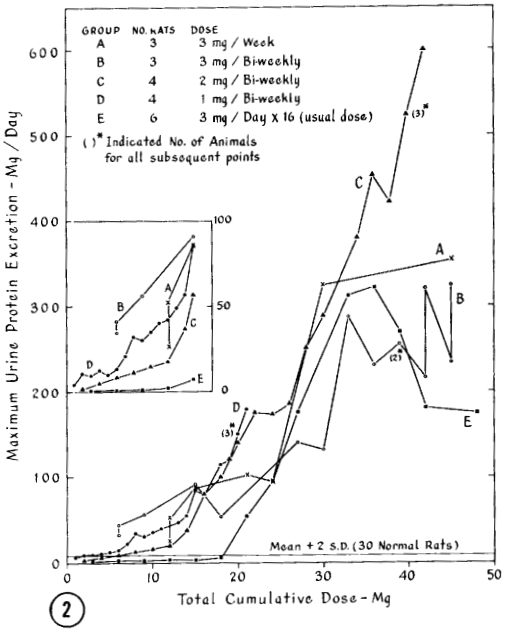
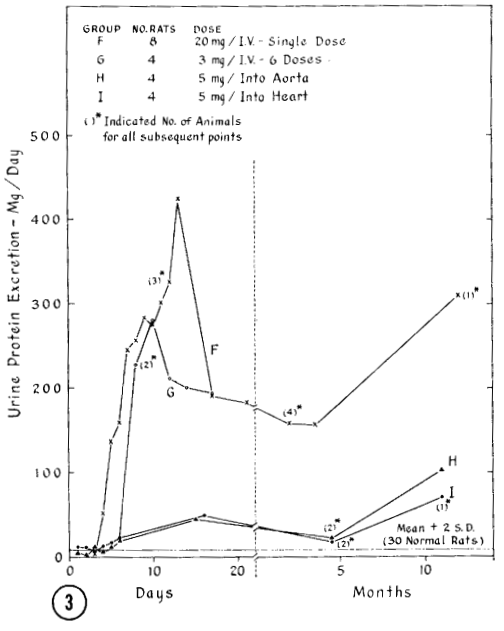
† 9-(3'-amino-3'-deoxy-B-D-ribofuranosyl)-6-dimethyl aminopurine.







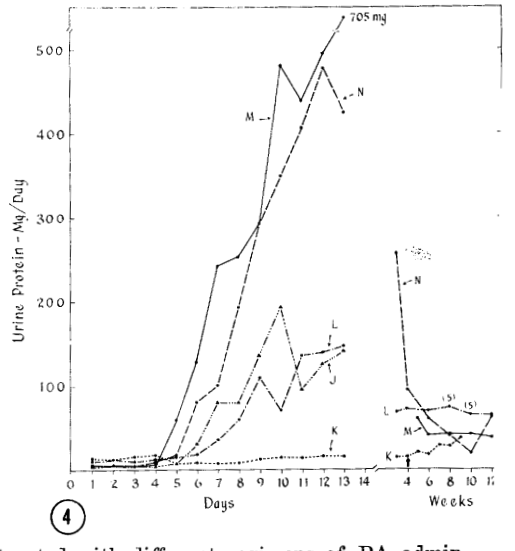


FIG. 1. Development of proteinuria in rats treated with different regimens of PA administered subcutaneously. The latent period is prolonged by giving 1-2 mg at biweekly intervals.

FIG. 2. Degree of proteinuria as related to total cumulative dose. From the inset curve with the expanded vertical scale it is evident that small doses given infrequently were more effective in producing proteinuria than the larger dose given daily.

FIG. 3. Development of proteinuria in rats injected with PA into circulating blood.

FIG. 4. "Stopped" schedules. Development and course of proteinuria in rats which received 1 to 4 daily subcutaneous doses of PA, compared to control rats which were treated daily for 2 wk. Curve J, daily × 13; K, single dose (repeated at 4 wk); L, daily × 2; M, daily × 3; N, daily × 4. Dose: 3 mg/rat/day. (5) = No. of rats.

Results. Subcutaneous PA administration at weekly and biweekly intervals. In Fig. 1 the mean daily protein excretion determined twice weekly is plotted against time. In Group A animals which received 3 mg of PA weekly, proteinuria appeared by the third week, then rose sharply to reach nearly 450 mg per day at 18 weeks. When the same dose was administered at biweekly instead of at weekly intervals (Group B) the rise in protein excretion with time was less dramatic but progressed at a steady rate. Decreasing the dose at this biweekly regimen caused the lag period to be prolonged considerably so that in Group C (2 mg biweekly) and Group D (1 mg biweekly) the degree of proteinuria remained low until the 12th week. Protein excretion then rose sharply in Group C to parallel Group B while the increase in Group D was more gradual.

Two of the 3 rats in Group A which received the largest dose died in the 21st and 23rd week, respectively, from renal insufficiency. The remaining rat died in the 27th week from renal insufficiency complicated by pneumonia. One of the 3 rats in Group B died with advanced kidney disease complicated by pneumonia during the 24th week; the other 2 rats were sacrificed in the 33rd week and their kidneys also showed advanced disease. One rat in each of Groups C and D which received the smaller doses died of pneumonia. The rest were sacrificed during the 48th week. Every kidney was enlarged and grossly diseased. It is interesting that 1 rat of Group D did not develop significant proteinuria until the 36th week after which protein excretion remained only slightly elevated above the normal range throughout the period of observation.

In Fig. 2 maximum daily protein excretion determined twice weekly is plotted against total cumulative dose for the same groups (A-D) and for Group E which represents data from rats given the standard dose of 3 mg of PA daily for 16 days. For Groups B, C and D which received PA in alternate weeks, 2 weekly values were available for a single dose and the graph is drawn through the mean of the 2 values. Protein excretion was always greater in the week following injection than during the week of injection.

The total cumulative dose required for induction of proteinuria by subcutaneous administration of PA *decreased* when the interval between doses was lengthened from daily (Group E), to weekly (Group A), or biweekly (Group B) (Fig. 2). This is quite apparent during the earlier phases (inset, Fig. 2) when the doses still fell below 15 mg. When the total cumulative dose approached 35 mg, all groups had marked proteinuria although the relative severity varied among the different groups. The leveling off of proteinuria in Groups A and B and the actual fall seen in the Group E animals *cf.* also Group J, Fig. 4) at higher levels of total dose probably reflect manifestations of reduced glomerular filtration and water retention present in these animals with the full-blown nephrotic syndrome.

Administration of PA into the circulating blood. The course of protein excretion following PA administration by the intravenous, intra-aortic, or intracardiac route is shown in Fig. 3. The initial lag period noted with subcutaneous PA administration is still apparent although it has been somewhat shortened from 5-6 days to 3 days in Group F which received the largest single dose (20 mg I.V.). The pattern of protein excretion of the Group G animals which received a total of 18 mg in 6 divided daily doses was initially similar to that of Group F but the peak excretion attained was somewhat less. Groups H and I which received only 5 mg injected into the aorta or heart, respectively, excreted much less, albeit significant, protein and the curves paralleled each other. Proteinuria persisted in survivors of Groups G, H and I and increased slightly with time.

Subcutaneous PA administration—"stopped" schedules. Proteinuria was induced even when daily subcutaneous administration of PA was stopped after 2, 3, or 4 successive days of treatment, and persisted in all surviving rats (Fig. 4). Animals of Group K which received a single 3 mg dose of PA did not develop proteinuria in 4 weeks of observation; however, protein excretion increased significantly in the weeks following the second challenge at 4 weeks.

The pattern of protein excretion in the rats which received only 2 doses of PA (Group L) was similar to that of rats which received the full course of 13 daily injections (Group J). This similarity was not evidenced clinically. Whereas 9 of the 12 rats in Group J died by the 4th week as a consequence of the acute nephrotic syndrome, the disease was hardly evident in the Group L animals, none of which even developed ascites. One rat in Group L died of pneumonia in the 8th week, but in contrast to the large, swollen kidneys noted in the animals of Group J, the kidneys of this animal appeared grossly and histologically normal except for an occasional protein cast in the proximal tubule. When PA administration was abruptly stopped after 3 and 4 successive daily doses (Groups M and N, respectively), urinary protein excretion remained low until 2 intervening days had elapsed. Proteinuria suddenly became quite acute rising to nearly 500 mg per day at the end of 12 days. Proteinuria still persisted in these animals 3 months after cessation of the drug (Fig. 4).

Summarizing these data, it can be seen that a "threshold dose" of approximately 4-5 mg‡ appears to exist, since a) single doses of 3 mg of PA administered subcutaneously did not give rise to significant proteinuria until the administration of a second dose of 3 mg at 4 weeks (Group K, Fig. 4); b) the lowest subcutaneous dose of 1 mg of PA given biweekly was effective only after the 4th dose (Group D, Fig. 2); c) at dosage schedules of 3 mg per week or 3 mg biweekly (Groups A and B, Fig. 1 and 2) the animals had already developed proteinuria when first examined on the 3rd week (unfortunately, urine protein values for the first 2 weeks were not available for these groups, but the data from Group K above are indicative of the probable result). It is important to point out that with subcutaneous PA administration proteinuria did not appear *until* this threshold dose was reached (Fig. 2, Groups C and D); however, once this dose was attained or exceeded proteinuria did appear, but only after a minimum latent period of 3 days (Fig. 1 and 4).

‡ For a 200 g rat.

Discussion. Buchanan(5) has been unable to demonstrate inhibition of any enzymes essential for *de novo* purine synthesis by puromycin, the parent compound from which PA is derived. However, puromycin does inhibit protein synthesis by rat liver *in vitro* and this block occurs at the later stages when the amino acid is transferred from soluble RNA to protein(6). Since this inhibition is instantaneous, the possibility appears remote that a metabolic fragment of puromycin, *i.e.,* PA itself, is acting as the true inhibitor. While Fisher *et al.*(7) found a decrease in cytochrome oxidase and succinic dehydrogenase in the proximal tubules and ascending loops of Henle in PA treated rats, this change was manifest only after the onset of proteinuria, and they concluded that the enzyme defects were probably secondary to the proteinuria and were not due to the direct action of PA. The same explanation may be offered for the decrease in alkaline phosphatase and increase in glucose-6-phosphate dehydrogenase activity found in glomeruli of PA-induced nephrotic rats(8). The experiments of Bartlett using mitochondria from liver and kidney of PA treated rats were inconclusive regarding the influence of PA on oxidative phosphorylation(9,10).

The appearance of proteinuria following administration of small amounts of rapidly excreted PA§ with relatively long intervals between doses as observed in the present experiments also argues against a mechanism involving *direct* enzyme inhibition. The minimum latent period of 3-5 days between the first administration of PA and the appearance of proteinuria likewise provides evidence against such a mode of action since proteinuria would be expected to appear almost immediately if enzymatic block were involved.

Wilson *et al.*(12,13) and Borowsky *et al.* (14) were able to lengthen the latent period, the latter investigators to 3 weeks by giving PA orally in the diet. By administering subthreshold doses subcutaneously at biweekly

§ Using 8-C^{14}-PA, it was found that 91% of the radioactivity of a single subcutaneous dose (3.0 mg/200 g normal rat) was excreted in the urine and feces within 8 hours(11).

intervals we were able to prolong the latent period to 3 months. In addition to influencing this latent period, the dose and route of administration of PA also affected the pathological development and severity of the disease(13,14,15). In general, lowering oral and subcutaneous doses led to a chronic state which often terminated in death from renal failure. There seems no doubt that once the drug has been administered in an adequate dose, the kidney damage is slowly progressive with gradual destruction of remaining functioning nephrons even though a relatively short course of treatment has been given. This suggests that the drug initiates a train of pathological events which is self-perpetuating even after the stimulus has been withdrawn, and which cannot be reversed by any means now known.

Other possible mechanisms by which PA might produce nephrotoxicity are by blocking incorporation of adenine(16,17,18) or adenosine(19) into nucleic acids, and participation in an immune mechanism(13).

Summary. Administration of 1-3 mg of PA subcutaneously to normal rats at intervals of 1-2 weeks produced significant proteinuria after a prolonged latent period provided the total dose exceeded a threshold dose of approximately 4-5 mg. Analysis of the cumulative dose-response curves revealed that doses administered subcutaneously at biweekly intervals were more effective in producing proteinuria than the same total dose given on a daily or weekly schedule. While the mechanism of PA nephrotoxicity is still not known, it is unlikely that it acts by *direct* enzyme inhibition.

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