The Effects of Guanidinosuccinic Acid and Methylguanidine on Erythrocyte Cation Transport[1] (36082)

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(Introduced by B. R. Forsyth)

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Reports by Welt *et al.* (1, 2) have indicated that there is a population of severely uremic patients (BUN > 100 mg/100 ml) whose erythrocytes have a high sodium content accompanied by a decrease in erythrocyte potassium influx and sodium outflux rate constants and ouabain-sensitive ATPase activity. Chronic hemodialysis of several such patients has been followed by a reversal of these defects (2). The alterations of membrane ATPase may be induced in normal cells by incubation in uremic plasma (3); however, Villamil *et al.* (4) could not induce any abnormalities of flux in red blood cells exposed to uremic plasma. Other workers have suggested that some monosubstituted guanidines are responsible for increased *in vitro* hemolysis of normal red blood cells (5) and for the production of uremic symptoms in dogs (6, 7). Horowitz and co-workers have found increased levels of guanidinosuccinic acid (GSA) in uremic plasma (8) and have noted an inhibitory *in vitro* effect of GSA on platelet factor III (9). Carr and Schloerb (10) have shown a tenfold increase in plasma methylguanidine (MGUA) in dogs, anuric for 4 days. Because of these studies, we tested the effect of GSA and MGUA on erythrocyte cation transport.

Methods. Solutions. The incubation solutions consisted of NaCl, 140 mM; KCl, 5 mM; sodium phosphate, 1.2 mM; $CaCl_2$, 1.5 mM; glycyl-glycine $MgCO_2$ buffer 54 mM and 10 mM respectively; glucose, 20 mM, bovine albumin, 100 mg/100 ml; adenine, 3 mM; and inosine, 10 mM. The proper amounts of guanidinosuccinic (GSA) and methylguanidine-HCl (MGUA) were added as water concentrates. Distilled water was added to the incubation medium to bring the osmolarity of all solutions to 295 ± 5 mOsm. The final pH was 7.40 ± 0.05. Bacterial contamination was avoided by either adding Keflin (sodium cephalothin, 20 μg/ml) to the solutions or by passing them through a Millipore membrane filter (0.45 μ) into sterilized flasks.

Incubation, Flux, and Cation Determinations. Fresh, heparinized human blood was incubated either directly or after triple washing with isosmotic $MgCl_2$. Enough red blood cells were added to the incubation medium to yield a hematocrit of 10–15% in 50 ml of suspension. Unless otherwise specified, all red blood cell samples were maintained in a water-bath shaker at 37° for 40 hr. After the incubation, the intracellular cation measurements and the sodium 22 transport experiments were carried out according to methods outlined previously (11, 12). In order to minimize changes of pH and osmolarity, the supernatant solutions were removed after 24 hr, by decanting or by sterile withdrawal with needle and syringe, and were replaced with fresh solution. The pH decreased to 7.25 ± 0.05 (SEM) after 24 hr and again after 48 hr. Blood agar cultures of samples taken at 48 hr showed no significant bacterial growth.

Results. Since the transport defect of uremic red blood cells is accompanied by an elevated sodium concentration (Na_c) and possibly by a diminished potassium concentration (K_c), we used Na_c and K_c as a screening test to assess the potential cytotoxicity of GSA and MGUA. Tables I and II show the results for Na_c and K_c, respectively, in 7 incubation experiments. No differ-

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TABLE I. The Effect of MGUA and GSA on Red Blood Cell Sodium (Na_c).

	Na_c after 48 hr incubation			Guanidine compounds
Zero time Na_c	Control	MGUA	GSA	conc (mM)
7.9	9.4	8.9	8.8	0.3
9.9	7.7	7.4	7.1	0.3
9.9	7.7	9.2	7.5	0.3
8.6	5.6	5.5	5.5	0.6
8.5	5.4	5.1	5.4	1.0
7.3	7.4	5.0	5.3	2.0
8.5	5.4	5.4	4.9	3.0
Mean ± SEM				
8.6 ± 0.4	7.0 ± 0.6	6.6 ± 0.7	6.3 ± 0.6	
p[a]		.9 < .8	.7 < .6	

[a] Nonpaired t test, MGUA and GSA vs control after 48 hr.

ences were demonstrated between control cells and cells exposed to the guanidine derivatives. In a single experiment, cells were incubated with combinations of the guanidine compounds and urea (147 mg/100 ml; 25 mM) and/or creatinine (20.3 mg/100 ml; 1.8 mM); however, no alterations of cation content were found.

Bidirectional tracer fluxes (sodium 22) were done to eliminate the possibility that the guanidines might inhibit a transport process which does not contribute to net flux and hence would not be identified with determinations of Na_c and K_c. Table III shows these data. No significant inhibition could be demonstrated for either the ouabain-inhibited or the furosemide-inhibited fractions of sodum outflux. Figure 1 depicts a typical outflux experiment after 48 hr exposure of normal cells to GSA or MGUA. The slope of the lines defines the outflux rate constants ${}^{o}k_{Na}$); and as shown, there were no appreciable differences among the 3 groups.

In addition two influx studies were done. Figure 2 shows one of these experiments after 48 hr exposure at 37° to MGUA and GSA. Another influx was done after 9 days exposure at 3°. Influx rate constants were similar for the 3 groups of cells in both experiments.

Discussion. Several laboratories have reported that a small population of uremic patients have an erythrocyte transport defect for sodium (2, 4) and potassium (1). These selected patients have a high Na_c, depressed sodium outflux rate constants and membrane

TABLE II. The Effect of MGUA and GSA on Red Blood Cell Potassium (K_c).

	K_c after 48 hr incubation			Guanidine compounds
Zero time K_c	Control	MGUA	GSA	conc (mM)
95.3	74.3	73.2	68.5	0.3
108.1	93.0	93.3	92.0	0.3
108.1	93.0	90.4	94.7	0.3
96.7	84.3	88.5	88.4	0.6
97.9	96.4	95.5	94.6	1.0
92.0	87.8	89.4	88.8	2.0
97.9	96.4	94.5	94.7	3.0
Mean ± SEM				
99.4 ± 2.4	89.3 ± 3.0	89.3 ± 2.4	88.8 ± 3.5	
p[a]		< .9	< .9	

[a] Nonpaired t test, MGUA and GSA vs control after 48 hr.

TABLE III. Effects of MGUA and GSA on Sodium Outflux.

Flux no.	Control			MGUA[a]			GSA[a]		
	Na_c	$\Delta^o k_{Na}^{I\,b}$	$\Delta^o k_{Na}^{II}$	Na_c	$\Delta^o k_{Na}^{I}$	$\Delta^o k_{Na}^{II}$	Na_c	$\Delta^o k_{Na}^{I}$	$\Delta^o k_{Na}^{II}$
1	11.3	0.260	0.008	10.8	0.202	0.010	10.4	0.206	0.009
2	7.7	0.300	0.058	7.5	0.292	0.065	7.4	0.274	0.057
3	59.0	0.154	0.030	59.8	0.150	0.028	58.9	0.159	0.022
4	8.0	0.213	0.074	6.8	0.249	0.064	6.9	0.229	0.083
p^c					$.7 < .6$			$.5 < .4$	

[a] Guanidine concentrations were 0.3, 0.6, 0.3, and 2.0 mM for fluxes 1–4. All incubations were 48 hr at 37° except flux 3 which was a 9 day incubation at 3°.

[b] $\Delta^o k_{Na}^{I}$ and $\Delta^o k_{Na}^{II}$ represent the decrement in the outflux rate constant produced by ouabain and by furosemide (in the presence of ouabain), respectively.

[c] Paired t test comparing control to MGUA- and GSA-treated cells.

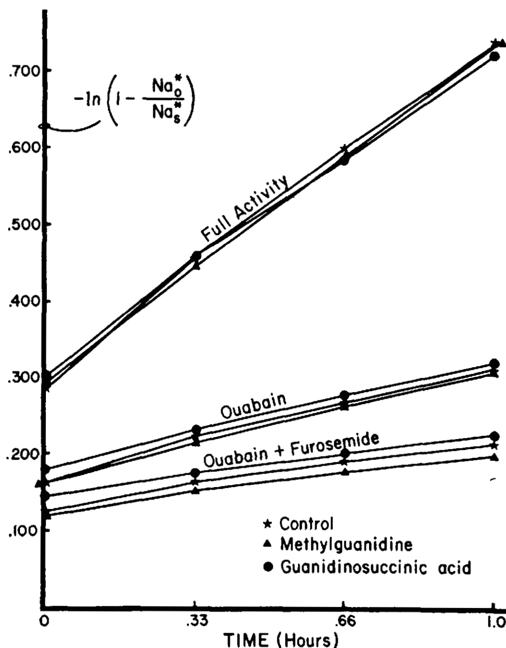

FIG. 1. This depicts a typical outflux study. The ordinate, — ln [1 — (Na_o^*/Na_s^*)], represents the cumulative appearance of ^{22}Na into the extracellular media. The specific values for the effects of ouabain and ouabain + furosemide on the outflux rate constants are shown in Table III, flux 2. It is apparent that MGUA and GSA had no effect on these fractions of outflux.

ATPase, and normal or high cellular ATP (1). The alterations appear to be slowly reversible with effective dialytic therapy (2). The other important observations, which laid the groundwork for the present study, were the investigations of Horowitz and co-workers (8, 9). These investigators have measured elevated levels of GSA (0.05 to 0.31 mM) in the blood of uremic patients (8) and have induced the "uremic type" of platelet factor III defect with GSA *in vitro* (8, 9). Lonergan *et al.* (13) have reproduced partially the uremic defect of erythrocyte transketolase activity by exposing normal cells to GSA. Giovanetti *et al.* have reported that several guanidine derivatives increase *in vitro* autohemolysis of human erythrocytes (5) and cause uremic symptoms in dogs (6). However Smith and Welt (14) found recently that incubation of healthy red blood cells with several guanidine compounds (GSA and MGUA were not tested) did not affect sodium outflux. Although Carr and Schloerb (10) have found elevated plasma levels of MGUA

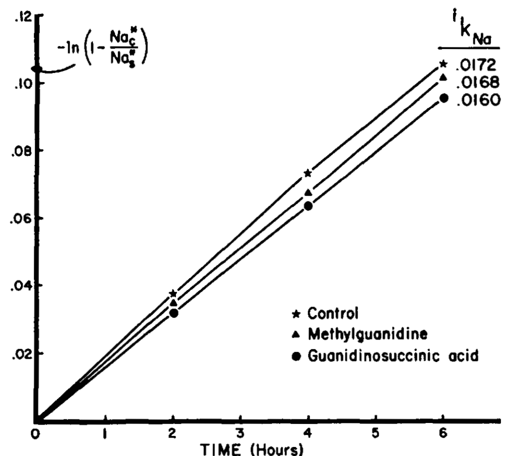

FIG. 2. This shows a representative influx. The ordinate, — ln [1 — (Na_c^*/Na_s^*)], defines the cumulative appearance of ^{22}Na into the cells (corrected for simultaneous loss of tracer due to outflux). MGUA and GSA had no appreciable influence on sodium influx.

in anuric dogs, Stein *et al.* (15) were unable to find any detectable increase in MGUA in the blood from 6 of 7 uremic patients even though urinary excretion of MGUA was increased in the uremic group. Wardle (16) has reported recently that many possible toxins of uremia have diverse effects on red blood cell enzymes, fragility, and metabolism. Guanidine, in concentrations of 50 mg/100 ml (uremic dogs have levels of 0.038 ± 0.024 mg/100 ml) was reported to increase potassium influx (16), contrary to previous work showing diminished potassium influx in uremic patients (1).

The present experiments diminish the likelihood that GSA and MGUA are important determinants of erythrocyte cation transport in uremic patients. The major limitations of our work are the length of incubation and the unknown synergistic role of other compounds besides MGUA and GSA. Since uremia in chronic renal failure develops slowly, it is possible that guanidines exert a deleterious effect on red blood cells over a period of weeks rather than days. Also our *in vitro* experiments simulate imperfectly the complex chemical environment of uremic plasma. The studies cited previously (5, 8, 9, 13), which have shown some cytotoxic effects of GSA *in vitro*, used concentrations in the range of 0.005 to 0.9 m*M* and incubation times no longer than 48 hr. We attempted to circumvent partially the limitations of the 48 hr incubation by using concentrations of GSA (up to 3.0 m*M*) substantially in excess of the highest recorded value in uremic blood (0.31 m*M* GSA) (8). In addition, our single 9 day incubation (Table III), which demonstrated no effects of GSA and MGUA, makes less likely the possibility of positive results with longer incubations. It might also be anticipated that if GSA alters red blood cell Na transport *in vivo* the defect of transport would appear more constantly since the elevations of plasma GSA are a predictable result of decreased renal function.

Summary. Cellular abnormalities observed in uremic patients, have been ascribed to toxins, hormones, or malnutrition. Guanidine derivatives, which accumulate in uremia, possess some toxic properties for platelets and erythrocytes. Accordingly *in vitro* effects of several guanidine compounds on cation transport in human erythrocytes were studied. Guanidinosuccinic acid (GSA) and methylguanidine (MGUA), in varying concentrations were incubated with normal human red blood cells. After incubations of 48 hr, the sodium and potassium content of erythrocytes exposed to these compounds did not differ from control cells. Sodium outflux and influx rate constants, determined with sodium 22, were unaffected by GSA and MGUA. We conclude that, under the conditions of these experiments, GSA and MGUA did not alter the steady state intracellular sodium and potassium content or the bidirectional sodium fluxes of human erythrocytes.

1. Welt, L. G., Sachs, J. R., and McManus, T. J., Trans. Ass. Amer. Physicians **77**, 169 (1964).

2. Welt, L. G., Smith, E. K. M., Dunn, M. J., Czerwinski, A., Proctor, H., Cole, C., Balfe, J. W., and Gitelman, H. J., Trans. Ass. Amer. Physicians **80**, 217 (1967).

3. Cole, C. H., Balfe, J. W., and Welt, L. G., Trans. Ass. Amer. Physicans **81**, 213 (1968).

4. Villamil, M. F., Rettori, M. F., and Kleeman, C. R., J. Lab. Clin. Med. **72**, 308 (1968).

5. Giovannetti, S., Cioni, L., Balestri, P. L., and Biagini, M., Clin. Sci. **34**, 141 (1968).

6. Giovannetti, S., Biagini, M., Balestri, P. L., Navalesi, R., Giagoni, P., De Matteis, A., Ferro-Milone, P., and Perfetti, C., Clin. Sci. **36**, 445 (1969).

7. Mason, M. F., Resnik, H., Minot, A. S., Rainey, J., Pilcher, C., and Harrison, T. R., Arch. Intern. Med. **60**, 312 (1937).

8. Horowitz, H. I., Stein, I. M., Cohen, B. D., and White, J. G., Amer. J. Med. **49**, 336 (1970).

9. Horowitz, H. I., Cohen, B. D., Martinez, P., and Papayoanou, M. F., Blood **30**, 331 (1967).

10. Carr, M. H., and Schloerb, P. R., Anal. Biochem. **1**, 221 (1960).

11. Dunn, M. J., J. Clin. Invest. **48**, 674 (1969).

12. Sachs, J. R., and Welt, L. G., J. Clin. Invest. **46**, 65 (1967).

13. Lonergan, E. T., Semar, M., and Lange, K., Arch. Intern. Med. **126**, 851 (1970).

14. Smith, E. K. M., and Welt, L. G., Arch. Intern. Med. **126**, 827 (1970).

15. Stein, I. M., Perez, G., Johnson, R., and Cummings, N. B., Clin. Res. **18**, 700 (1970).

16. Wardle, E. N., Acta Hematol. **43**, 129 (1970).

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