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Alleviation of mortality induced by salicylate and stress

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Abstract. Protection from the deleterious effects of the interaction of environmental stress and salicylate by calcium supplement was investigated in 96 pigmented rats. Within a 2 × 2 × 4 factorial design, rats were assigned to groups defined by: *A*) ad lib access to 1) plain tap water, or 2) 50 mM calcium chloride solution; *B*) exposure to stressors consisting of daily 10 h periods of 1) 98 dB SPL noise, or 2) confinement precluding movements; *C*) daily injections of 233, 350, or 410 mg/kg of sodium salicylate or the saline vehicle. For subjects maintained on tap water, weight loss and mortality increased with salicylate levels, with all subjects dying in the group drinking water and injected with 410 mg/kg. Calcium protected all of the subjects in the noise stress group but not in the confined group.
Key words. Salicylate; noise; mortality; weight; tinnitus; rats.

Aspirin 'the wonder drug', is probably the most commonly used medication worldwide[1]. Its application spans a range of medical problems and symptoms, and its clinical benefits are enormous. In the majority of applications, prescribed dose levels do not induce significant side effects, except for stomach irritation, and, at higher doses, 'ringing in the ears' – tinnitus – neither of which is life threatening[1]. The regularity of the emergence of salicylate-induced tinnitus has been sometimes used to titrate the dose of aspirin in patients with rheumatic arthritis[2]. Recent data indicating that daily ingestion of salicylate may decrease the changes of heart attack[3–5] have further increased the popularity of aspirin and its consumption.

For our work on the mechanism of tinnitus, we have induced it in animals through s.c. injection of sodium salicylate[6–8]. From a functional point of view, other salicylates have the same effect as acetylsalic acid – aspirin[1], and, at any rate, aspirin is rapidly converted in the body to salicyclic acid, with a half life of 20 min[9,10]. Experimental studies involving well over 500 rats have evaluated behavioral changes after exposure to mildly intense noise (62 dB SPL) and administration of sodium salicylate. We have noticed that although the mortality rate is very low, it does nevertheless occur exclusively in groups exposed to noise and injected with salicylate. At first glance, the notion that salicylate interacts with noise and is involved with mortality in experimental animals seems bizarre, since the most serious impact that has been postulated from such an interaction is an enhancement of temporary or permanent hearing loss[11,12]. We decided to explore the effects of the interaction of noise and salicylate in a systematic manner through a parametric study using all combinations of different salicylate doses and noise intensities to assess survival rates and weight changes in rats. The results of this study[13] revealed that, while salicylate alone or noise alone are without impact on a subject's well being, their interaction is literally deadly. Furthermore, mortality and weight loss are systematically dependent on the salicylate dose/noise intensity levels. The mechanism of this action remains elusive, and detailed pathological examinations revealed no basis of any sort for the mortality or any other abnormality that might indicate presence of any lethal problems[13].

Since our report, this finding has been confirmed by two independent groups[14,15]. Mindful of the popularity of salicylate consumption and the very common exposure to intense noise in the work place and society in general, we wanted to continue work on this phenomenon even without knowing the specific mechanism responsible for it, in order to search further for a possible means for its alleviation.

One obviously possible mechanism underlying the interaction between salicylate and noise is related to stress—i.e. suggesting that intense noise induces stress in rats, and salicylate acting upon the organism under stress has a fatal effect. Such a possibility seemed plausible, even if quite nonspecific, since stress causes a wide range of metabolic and hormonal changes, which might affect the organism's susceptibility to potentially toxic effects of salicylate. This possibility seemed worth exploring, given the extent to which stress pervades society, as well as the likelihood that the toxic effects of salicylate might overlay a stressful background, further expanding the proportion of the population affected. Therefore, the report describes attempts to evaluate whether it is possible to associate salicylate-related mortality with presumably mild stress from either induced sound or, alternatively, partial restraint of an animal's movement. The second aim of this study was to explore a possible method for alleviating instances of mortality by provid-

ing subjects with exogenous calcium, an approach that has shown some success in preventing the teratogenic effects of salicylate in rats[16,17] and in attenuating salicylate-induced tinnitus[18,19].

Materials and methods

Subjects. A total of 96 male hooded rats derived from Charles River strains and bred at the University of Massachusetts at Boston, 120–180 days old and weighing an average of 352.09 g (SEM = 7.69), were housed individually for the duration of the experiment in standard laboratory cages (Fenco Products) measuring 43 cm long × 25 cm wide × 17 cm high. Three sides of each cage were made of solid stainless steel, and one long side facing out from the housing rack was made of stainless steel wire mesh which supported a food storage bin and a water bottle. Individual cages were stored in one of two single-sided racks 1.5 × 2 m, accommodating 5 rows of 3 cages. Each rack was housed singly in acoustically shielded cubicles, 3.5 × 4 m, separate from the main rat colony. Food and water were available ad libitum in the home cages, and subjects were maintained on a 12 h light/dark cycle throughout the experiment.

Apparatus. Background noise levels were provided by a Hewlett Packard precision Noise Generator (Model 8057A) and delivered through a 9 cm wide Motorola speaker (P35-VAH) placed on a table located 1 m in front of the center of the animal storage rack. In order to obtain a sound field as consistent as possible with the previous study[13], sound pressure levels, recorded near the center of the storage rack, were measured by a Brüel-Kjaer Precision Sound Level Meter (Type 2203) at 55 dBA (the ambient background level) and 101 dBA, the most intense noise level used in the previous study. Sound pressure levels were approximately the same for all cages. Since hearing threshold curves of rats differ significantly from those of humans, rats having higher thresholds at low frequency regions and lower thresholds for high frequencies[20], SPL noise levels were recalculated from levels determined for frequencies of 1–16 kHz in 1/3 octave bands (Quest Electronics, Impulse Precision Sound Level Meter, Model 155, and 1/3 Octave Band Filter, Model OB-133) according to standard procedures[21]. The calculated levels were 0 and 98 dB SPL for the 55 and 101 dBA levels, respectively. In the remaining text, the noise level used as a stressor will be referred to as 98 dB SPL. The ambient, no experimental noise condition was below the hearing threshold of rats[20].

For the confinement periods, a total of 12 small chambers were constructed of wire mesh and wood lined with contact paper for ease in cleaning. The chambers were attached in groups of four, and one end and the bottom of the grouping was made of wire, while the other end and hinged lids were constructed of wood. The average internal dimensions of the chambers were 5.19 cm wide × 14.17 cm high × 22 cm long. The chambers were suspended 3 cm above a tray of laboratory grade wood shavings for collection of waste during the 10 h daily periods of confining subjects.

Half of the total numbers of subjects were assigned to a calcium treatment in which their ad lib drinking was maintained on a 50 mM solution of calcium chloride, mixed freshly each day. The remaining 48 subjects continued with tap water in their home cage bottles, and these were freshly filled each day as well. Total calcium level in the tap water measured 0.0425 ± 0.0094 mM ($n = 8$). All subjects were weighed daily between 8:00 a.m. and 8:30 a.m. and injected s.c. with the appropriate salicylate dose or saline immediately afterward, and the amount of the injection depended on the actual daily weight. Salicylate dose levels of 233, 350 and 410 mg/kg of sodium salicylate, dissolved in saline, corresponded to three dose levels 200, 300, 350 mg/kg of salicylate acid, and saline only. Thus in this study, each subject was assigned to one of four drug treatments, and the salicylate acid levels will be used to describe a given group rather than sodium salicylate doses for the remainder of this report.

Half of the subjects under each drinking solution regimen and salicylate dose treatment were exposed daily to a noise level of 98 dB SPL, and the remaining 48 subjects, distributed over drinking solution and salicylate treatments, received daily confinement. Both stressor conditions of noise or confinement began daily at 08:00 h and lasted until 18:00 h. Subjects were removed from the confinement chambers in the order in which they were placed and returned to the home cage. This schedule of noise exposure or confinement continued for up to 15 days. Gross pathological examinations were conducted on subjects that expired during the experiment, and blood samples were obtained from part of the remaining subjects after the experiment was completed to evaluate serum salicylate levels[22]. Daily body weights were analyzed by means of analyses of variance for repeated measurements followed by Duncan tests[23,24].

Results

The overall results of this experiment confirmed that higher doses of salicylate interact with environmental stress to produce dramatic rates of mortality. Equally striking, moreover, was the finding that a diet with enhanced calcium content seems to have afforded protection from the lethal effects of noise stress and salicylate, even at the highest level. As may be seen in table 1, at the highest salicylate dose level of 350 mg/kg, all subjects drinking tap water died within the 15 days of experimentation, while in the group given the calcium solution and exposed to noise, all rats survived. Within the 300 mg/kg salicylate treatment, 5 out of 6 subjects drinking plain water and exposed to noise died during

Table 1. Numbers of fatal cases in groups of 6 animals each during 15 days of the experiment for rats exposed to one of four salicylate doses, given confinement (Confine) or noise stress, and provided access to 50 mM calcium in the drinking water or plain tap water.

Liquid diet	Stress	Salicylate (mg/kg) 350	300	200	0
Water	Noise	6	5	0	0
Water	Confine	6	1	0	0
Calcium solution	Noise	0	0	0	0
Calcium solution	Confine	6	2	0	0

the 15 days of injections, one subject given drinking water and confined died and two subjects given the calcium solution and confined died, but there were no deaths among subjects given calcium and exposed to noise. None of the subjects drinking calcium or plain water died after receiving either saline or 200 mg/kg salicylate injections under either the noise or confinement treatments. In summary, there was increasing mortality with salicylate dose increases under both types of stress, which was eliminated under noise stress if subjects had free access to the calcium solution. The protective effect of calcium in groups exposed to noise was confirmed by the significant dependency between the solution variable and the type of stress (Chi Square[1] = 8.47, $p < 0.004$), but no other statistically significant differences were found.

Daily consumption of fluid was monitored throughout the experiment, and the group means and related SEMs are presented in table 2. ANOVA of individual consumption did not reveal any systematic effects from salicylate dose, fluid consumed, or type of stressor, confirming the impression from table 2.

As in our earlier study[13], weight loss proved to be a sensitive index of effects from the interaction of salicylate injections and noise. Figure 1 shows the absolute weight change from pre-experimental levels until day 15 (or the animal's death) for each group drinking water or the calcium solution and exposed to noise or confinement at each of the salicylate dose levels. As figure 1 indicates, there were dramatic weight losses found in subjects drinking normal tap water and exposed to noise stress for the salicylate doses of 300 and 350 mg/kg, or exposed to confinement stress and given 350 mg/kg of salicylate. In those groups with mortality occurrences, the mean and SEM for the percent weight loss in animals that died were:

- water - noise - 350 mg/kg, -4.5% (± 1.0);
- water - noise - 300 mg/kg, -3.2% (± 0.8);
- water - confinement - 350 mg/kg, -7.1% (± 1.5);
- water - confinement - 300 mg/kg, -8.9%;
- calcium - confinement - noise - 350 mg/kg, -4.3% (± 1.3); and
- calcium - confinement - 300 mg/kg, -5.9% (± 7.9).

Subjects at those dose levels, but maintained on the calcium solution, had much less weight loss. The only exception to the trend between salicylate and weight loss, within the calcium treatment, is seen at the 200 mg/kg dose level and confinement stress. This relatively weak level of salicylate has been found in other studies to be at or just above threshold in terms of salicylate-induced tinnitus[25]. The dramatic average weight loss in the confinement group maintained on calcium is due to two of the subjects showing dramatic loss, although in neither case was the weight loss lethal.

Analysis of the mean weight change data confirmed the impression from figure 1. The main effect of ad lib drinking solution, water or calcium, attained significance ($F[1/80] = 4.95$, $p < 0.03$) as did the main effect

Table 2. Each cell shows the average volume (mls) of water or calcium 50 mM solution consumed by each group as well as the related SEM and the number of measurements (in parenthesis).

Liquid diet	Stress	Salicylate (mg/kg) 350	300	200	0	mean
Water	Noise	7.1 ± 1.4 (55)	6.4 ± 2.0 (74)	8.4 ± 2.9 (90)	7.7 ± 2.4 (90)	7.4 ± 1.3 (309)
Water	Confine	5.9 ± 1.6 (70)	6.8 ± 1.8 (87)	6.2 ± 2.1 (90)	7.3 ± 1.8 (90)	6.6 ± 1.1 (337)
Calcium solution	Noise	6.3 ± 1.4 (90)	7.5 ± 2.1 (90)	5.8 ± 2.1 (90)	6.1 ± 1.5 (90)	6.4 ± 1.2 (360)
Calcium solution	Confine	6.7 ± 1.6 (58)	8.1 ± 2.8 (79)	8.0 ± 2.5 (90)	7.9 ± 2.0 (90)	7.7 ± 1.1 (317)
Mean		6.5 ± 1.2 (273)	7.2 ± 1.6 (330)	7.1 ± 1.9 (360)	7.3 ± 1.0 (360)	7.0 ± 0.5 (1323)

The means were calculated by adding daily consumption of all surviving rats on a given day during the 15 days of the experiment and dividing by the total number of measurements. Thus, given different survival rates (see table 1), the number of measurements differed for various groups. Confine = confinement.

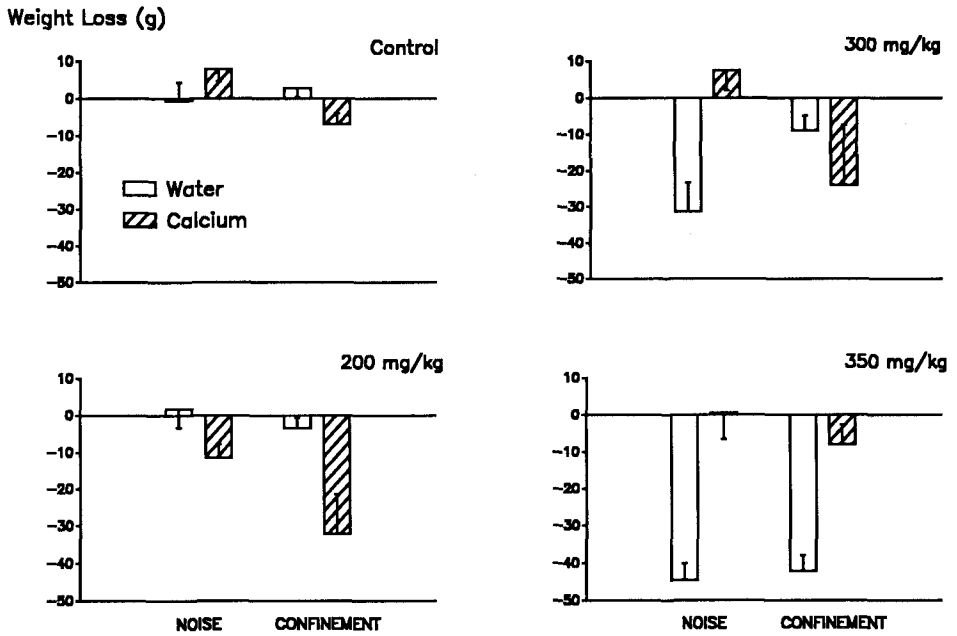

Figure 1. Mean weight loss at 15 days and SEMs for groups drinking plain water (open bars) or the 50 mM calcium chloride solution (diagonal bars) and exposed to noise or confinement stress at each of the salicylate dose levels ranging from the control (saline vehicle only) to 350 mg/kg. Each group initially consisted of 6 animals.

from the type of stress, noise or confinement, ($F[1/80] = 3.85$, $p = 0.0534$) and their interaction effect ($F[1/80] = 13.52$, $p < 0.0004$). The main effect of salicylate dose was highly significant ($F[3/80] = 8.99$, $p < 0.0001$) as was the interaction between dose and drinking solution ($F[3/80] = 14.28$, $p < 0.0001$). Neither of the remaining interactions of dose × stressor ($F < 1$) nor dose × stressor × drinking solution ($p = 0.097$) attained acceptable significance. Duncan tests (all p values < 0.05) revealed that subjects drinking water lost more weight than rats drinking the calcium solution, and the confinement tended to result in greater weight loss than did the noise stress. The Duncan tests also showed that weight loss in the saline subjects was less than in all other subjects. Weight loss in the 200 mg/kg dose level was less than in the 350 mg/kg subjects, but did not differ significantly from the 300 mg/kg dose level. Weight loss was not different between the 300 and 350 mg/kg dose levels, probably reflecting a saturation stemming from the high instances of mortality at these levels.

Figure 2 depicts the weight loss over experimental days for those groups in which mortality occurred. It is very clear from this figure that the subjects drinking the calcium solution and exposed to noise at both the 300 and 350 mg/kg dose levels were immune to the profound weight losses of the other groups. That this protection by calcium is specific to the auditory modality is evidenced from the lack of any clear calcium effect in the groups that were confined for 10 h each day.

All expiring rats died during the night, when they were not under the influence of salicylate or were not exposed to either noise or confinement stress. Upon discovery of a mortality case, the subjects were checked for any nasal or anal bleeding, and then the abdominal cavity was surgically opened to inspect visually for ulcers. The general post mortem examinations of the subjects that had expired did not reveal any gross pathological abnormalities that could obviously account for the instances of mortality.

Discussion

The main results of this experiment showed that:

1) ad lib drinking of the calcium solution protects subjects from deadly effects of interaction of intense noise and salicylate,

2) calcium did not protect subjects stressed by confinement, and

3) subjects drinking plain water during the experiment succumbed to the lethal interaction between high dose levels of salicylate and both types of stressor.

The latter finding replicates our earlier work[13] as well as that of others[14,15]. That the present results were obtained from male subjects, while our previous study was conducted with female subjects, suggests that the salicylate interaction with environmental stress is not gender specific. The mechanism underlying the calcium effect is still unknown, but might be related to stress and to calcium homeostasis.

The auditory-related stress effects can be reversed by calcium, but stress-related effects to physical restraint are seemingly not relieved. One of the problems in interpreting the contrast between calcium action on the two sources of stress in this study concerns the issue of

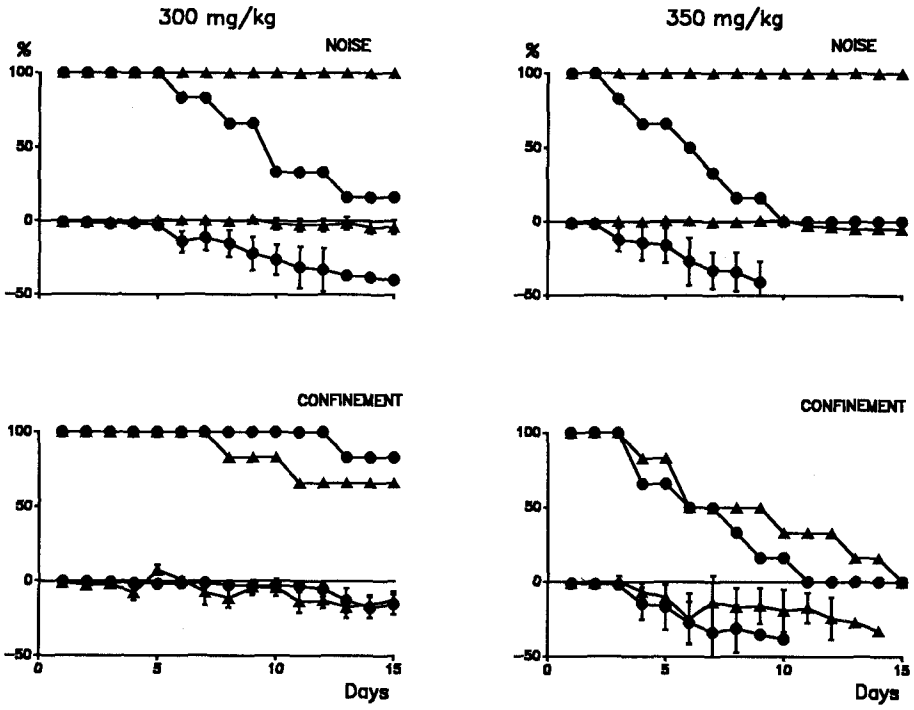

Figure 2. Relative mean weight loss and SEMs across experimental days for groups where mortality was observed under noise or confinement stress. In each figure, the top curves indicate the percent of surviving subjects out of 6 animals in each group for a given experimental day. The lower part of each figure shows mean weight change in the surviving subjects. Circles indicate subjects drinking plain water throughout the experiment, while the triangles indicate subjects drinking 50 mM calcium chloride solution.

the equivalence of the two stressors. While these data do not entirely eliminate the question of whether one stressor is weaker than the other, the fact that both stressors resulted in mortality at high dose levels suggests one type of functional equivalence. This equivalence neither implies that stress induced by these two stressors is exactly the same nor that they have the same susceptibility to additional experimental manipulations. Indeed, it would be surprising if they happen to induce exactly the same level of stress. The point is that the data suggest the interaction of salicylate with any stress as a basis for observed effects.

As in our earlier study[13], weight loss provided some correlate of mortality. The analysis of weight change in individual rats did not reveal clear correlations between weight loss and mortality. Group averages, while interesting as estimates of the probability of mortality occurrence, are not representative in understanding the mechanism for mortality. Thus, weight loss is not responsible for mortality. Interestingly, the weight loss resulting from the interaction of noise and tinnitus-inducing agents has been proposed as an animal model for tinnitus research[26], postulating that tinnitus-induced stress might contribute to an animal's mortality. Further studies are needed to clarify this hypothesis.

Several conclusions may be drawn from these data. There is a strong indication of salicylate-induced tinnitus, which somehow involves calcium. Our data on tinnitus[18,19] are consistent with the postulate that salicylate-induced decrease of the cochlear-free calcium is involved with the production of tinnitus. We were able to attenuate salicylate-induced tinnitus by exogenous calcium supplement[18]. Calcium supplement has been reported to alleviate teratogenic and analgesic effects of salicylate as well[17,27]. Therefore, it seems possible that a calcium supplement is partially reducing the action of salicylate on the organism.

The simplest, although general, explanation of our results involves stress induced by noise exposure or by mild restraint of movements. The stress induced by these methods may differ in its impact on hormonal and metabolical balance, which results in different susceptibility to the toxic effects of salicylate and to the protective effect of calcium supplement. Our results indicating a lack of protection from mortality by exogenous calcium are consistent with the hypothesis that confinement-induced stress is stronger than stress resulting from noise exposure so that partial alleviation of the salicylate effect by exogenous calcium is not sufficient to prevent mortality. Indeed, it may be a third source of stress, from salicylate-induced tinnitus, which adds to the stress induced by noise or confinement, resulting in mortality and weight loss[13,26]. Further work is needed to clarify: 1) whether hormonal imbalance is involved, 2) the specificity and level of stress, and 3) which physi-

ological parameters different from weight loss correspond to mortality.

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