activity of the pump increases in the stimulated tissue. Our data reveal several characteristics of this K uptake during postnatal glandular development and also indicate that it occurs in part by a furosemide-sensitive transport system in later stages of postnatal life and in the adult animal.

In resting (unstimulated) tissue, K uptake appears to be smaller and not inhibited significantly by ouabain in the first few days of life. This suggests that the ouabain-sensitive pump has a relatively low resting activity during this early period of postnatal gland development. Morphologically, the rat submandibular gland consists mostly of terminal tubular and proacinar cells at this period[9-11] and it is possible that these precursor cells have a pump with a resting activity lower than that of the fully differentiated salivary cells of later developmental stages. Our data indicate, however, that the ^{42}K space (i.e., K uptake) was significantly enhanced by carbachol and that this effect was uniformly inhibited by ouabain at all ages studied. This suggests that the Na, K pump can be activated by cholinergic stimuli from very early phases of postnatal development, as previously suggested[1,12,13].

Our results also indicate that part of the secretagogue-stimulated K uptake occurs by way of a furosemide-sensitive transport system after approximately 3 weeks of life (table 2). This transport system does not seem to be active in the absence of stimulation at any of the ages studied and although present in the glands of early postnatal animals does not become fully responsive to the cholinergic stimulus until later in development. These findings support the view that there is a temporal dissociation in the appearance of two major ion transport systems in the rat submandibular gland during postnatal development[1]. A secretagogue-activated Na, K pump is present from the immediate postnatal period, while a furosemide-sensitive transport system which moves Na, K and Cl across the salivary cell membrane does not become fully responsive until later in development. As both the Na, K pump and the furosemide-sensitive co-transporter are thought to participate in the formation of saliva[2-4,7], this dissociation is likely to influence the ability to secrete during the early phases of postnatal gland development[14].

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Restraint stress induced changes in rat liver and serum metallothionein and in Zn metabolism

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Summary. 24 h of a psychogenic stress (restraint) caused a strong increase of liver metallothionein (MT) levels. 3 h of stress were sufficient to induce an increase in liver MT, measured 21 h later, but the increase was much lower than in continuously restrained rats. Stress induction of liver MT was not due to food deprivation, since rats deprived for 24 h showed lower MT levels than stressed ones. Zn on MT presented the same qualitative but not quantitative pattern of response as MT protein. Liver cytosolic Zn was increased by restraint in spite of their being no decrease in serum Zn. Any treatment altered serum MT. Liver and serum MT were not correlated. The present results demonstrate that basically psychogenic stresses increased liver but not serum MT levels. No positive evidence for a relationship between corticosterone secretion and MT induction was found.
Key words. Metallothionein; pituitary-adrenal; zinc; restraint stress; copper.

Metallothioneins (MT) are low molecular weight, cysteine-rich, heavy metal-binding proteins. They are distributed among several species and organs, in which their synthesis is induced by the metal ions to which they bind, i.e. Cd, Zn and Cu[1]. Although its physiological function(s) remains to be established, it has been hypothetized that MT is related mainly to Zn and Cu metabolism[2]. In addition, it has been reported that some stress stimuli increase liver[3-10] and serum[8] MT. However, previous studies of the effect of these stimuli on MT had serious limitations: a) These stimuli could be considered mainly as physical stressors. Since it is believed that the term stress must be restricted to those stimuli which have mainly a psychological component[11], the effect of a psychogenic stress on MT levels remained to be established. b) No attempts were made to determine whether or not a continuous exposure to the stress stimulus for several hours was necessary to induce MT synthesis. c) No attempts were made to separate the effect of these experimental manipulations per se from their effect on food intake. This seems important since these stimuli might alter food intake[12], and deprivation increases hepatic MT[8]. d) Liver MT levels were measured indirectly using different methods[13-19]. It is therefore difficult to compare quantitatively the results obtained by different authors and their relevance with regard to stress-induced MT synthesis. Furthermore, because of the lack of specifity and sensitivity of these methods MT levels in serum were not measured in most studies. e) Glucocorticoids have been related to MT regulation both in vivo[20-23] and in vitro[24-26] studies, but reports are also conflicting[3,27]. To give insight into the relationship between glucocorticoids and MT, pituitary-adrenal hormones and MT were studied jointly in normal and stressed rats. Recently, a highly specific and sensitive radioimmunoassay method for MT has been developed[28-30]. It is the purpose of the present work to study the effect of a psychogenic stress (restraint) on liver and serum MT as well as on pituitary-adrenal hormones and reevaluate the relationship between MT and Zn metabolism during stress.

Materials and methods. Animals. Female Sprague-Dawley rats (85 ± 6 days old) were housed in groups of four per cage in a controlled room (light on 7–19 h, temperature 22 °C, white noise constant) for at least one week before starting experiments. Food and water were available ad libitum.

Procedure. At 09.00 h the animals were randomly assigned to the following experimental groups: A) Control: rats left undisturbed in the animal house and killed at the same time as re-

strained rats. B) Restraint: rats exposed to restraint stress in plastic tubes (6 × 20 cm), provided with several holes, for 0.5, 3, 8 and 24 h. All rats were restrained at 09.00 h and killed after different periods of stress. The stress environment was a room with the same temperature, illumination and background noise as the animal house. C) Restraint+resting: some rats stressed at 09.00 h were removed from the tubes 3 h later and then returned to their home cages. They were killed 5 or 21 h later, at the same time as the other experimental groups. D) Water and food deprivation: rats left undisturbed in the animal house but without food and water available. They were killed 8 and 24 h later at the same time as the appropriate control groups. Water and food deprived rats were killed only after 8 or 24 h of deprivation since rats eat primarily in the dark phase of the circadian rhythm[31]. The animals were killed by decapitation within 30 s after being taken from the animal house or the stress room, in an area adjacent to both rooms. The blood from the trunk was collected in plastic tubs at 4 °C to prevent corticotropin (ACTH) and MT degradation. Blood was centrifuged at 4000 rpm for 10 min at 4 °C and the serum obtained was frozen at −20 °C. Immediately after decapitation the liver was removed, weighed and frozen at − 90 °C. At the appropriate time, the livers were thawed and homogenized (2 v/w) in ice cold 10 mM Tris HCl, pH 8.2, containing 0.25 M sucrose and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 50,000 × g (15 min, 4 °C). The supernatant was lyophilized and stored at −20 °C until assayed.

Assays. ACTH was analyzed by radioimmunoassay using a commercial kit (Cea-Sorin). Serum corticosterone was assayed by radioimmunoassay as previously described[32], using a highly specific antiserum kindly provided by Dr A. Aldujaili which has very low cross-reactions with progesterone[33]. MT radioimmunoassay of cytosols and sera was performed as previously described[28–30]. Standard curves (inverse variance weighted logit-log regressions) were developed for cytosols and for 100 µl aliquots of serum. The ^{125}I label was on rat MT-1; the competitor was a 50/50 mix of rat MT-1 and rat MT-2. All unknowns were measured in quadruplicate, plus duplicate measurement of sample non-specific bound values.

Determination of Zn on MT. Zn levels on MT were analyzed after selective acetone precipitation, since MT precipites in 60–80% acetone[6]. Usually, acetone was added to 700 µl of 50,000 × g cytosol to give 60% acetone, centrifuged (4000 × g, 30 min) and the supernatant made up to 80% acetone and centrifuged again. Supernatant was discarded, and the precipitate resuspended in 2 ml of distilled water. All steps were performed at 4 °C. Some parallel samples (4 ml of 50,000 × g cytosol) were applied to Sephadex G-75 columns (1.5 × 85 cm), which were preequilibrated with 10 mM Tris HCl, pH 8.2, and eluted with the same buffer at a flow rate of 30 ml/h at 4 °C. Fractions (4.7 ml) were collected and monitored for absorbance and Zn and Cu levels. The Zn levels found in the MT peak (Ve/Vo = 2.0–2.5) were compared to those obtained with the selective acetone precipitation, showing that the Zn recovery on the 60–80% acetone precipitate was about 70% from that of Sephadex G-75 MT peak levels.

Measurement of metal levels. Zn and Cu were measured by atomic absorption spectrophotometry in a Perkin-Elmer 703 spectrophotometer. MT preparations and column fractions were aspirated directly into the air-acetylene flame. Metals from cytosol and serum were measured in the same way after diluting them 10 and 5 times respectively.

Statistical analysis. Results were analyzed with the Student t-test or with ANOVA after logarithmic transformation of the data to achieve homogeneity of variances. Some a priori comparisons were programmed: a) Two-way ANOVA with stress and time as factors in which only A and B groups were used. b) One-way ANOVA (or Student t-test) for comparisons of the different groups killed at the same time. In these cases the Duncan procedure ($p < 0.05$) was used for further comparisons between groups after one-way ANOVA. Pearson coefficient correlation between different variables was also calculated using logarithmic transformed data.

Results and discussion. Figure 1 shows the typical elution profiles found after Sephadex G-75 chromatography of samples from the control and from the 24-h restrained rats. As expected, only trace levels of Zn were detected at the MT elution position in samples from control rats, whereas those from stressed rats had about one third of the total Zn applied to the columns in the MT peak. No differences were observed in Zn associated with high molecular weight proteins. Copper was also present in the low molecular weight protein peak from the stressed rats, but the Cu peak eluted consistently just before the Zn peak. This suggests that the two metals are bound to different protein molecules. This is in contrast to other physiological situations, such as in fetal liver MT[34] or after MT induction by metal salts[35] or food restriction[4], where Cu and Zn peaks are coeluting in chromatography. Whether or not all these metal-containing proteins are MT remains unknown, and no further attempts were made in this study to identify the Cu-binding molecules. In any case, the Cu levels found in MT chromatographic fractions were very low and, therefore, MT was essentially present as ZnMT in restrained rats.

Liver MT levels and Zn content as MT are depicted in figures 2A and 2B, respectively. Two-way ANOVA revealed a significant effect for both stress and period of stress on MT levels ($p < 0.002$). One-way ANOVA revealed significant differences in liver MT between the 24-h experimental groups ($p < 0.0001$). The Duncan procedure revealed that MT levels in each of the four groups (control, restraint, restraint plus resting and de-

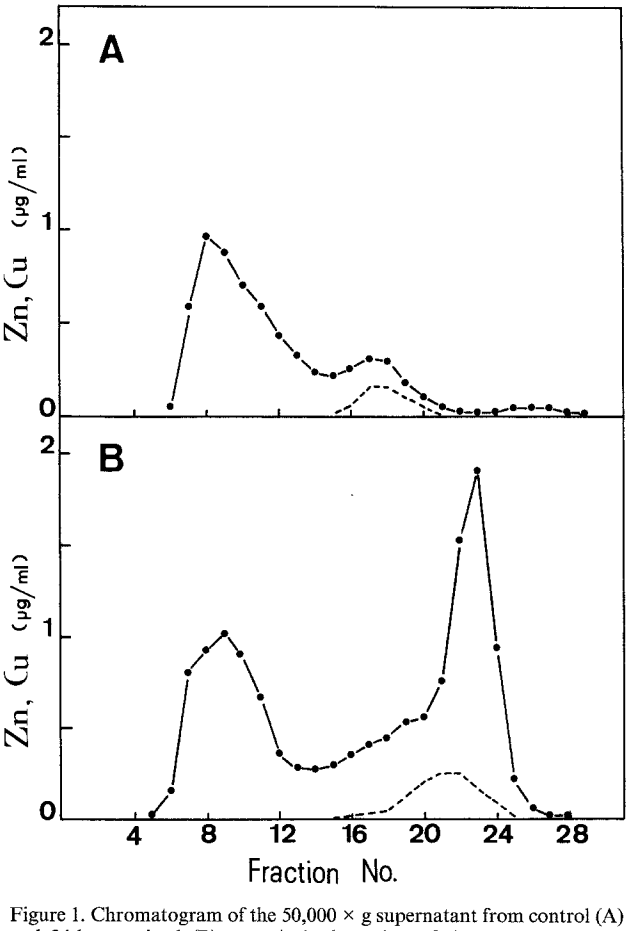

Figure 1. Chromatogram of the 50,000 × g supernatant from control (A) and 24-h restrained (B) rats. A 4-ml portion of the supernatant was applied to Sephadex G-75 columns (1.5 × 85 cm) and eluted with 10 mM Tris HCl, pH 8.2 at a flow rate of 30 ml/h at 4 °C. Fractions (4.7 ml) were collected and monitored for Zn and Cu levels. (●—●), Zn; (---), Cu.

prived) differed significantly from MT levels in the other three groups ($p < 0.05$). Earlier studies on the stress effect on liver MT showed that 24 h of cold stress, 3 h of strenuous exercise and CCl_4 injection caused an increase of liver MT of 315%, 302% and 543%, respectively, as measured by ^{65}Zn incorporation on MT[5]. By contrast, 24 h of restraint stress produced an increase in liver MT of 1742% as measured by MT RIA, and of 846% as measured by Zn on MT. Therefore, in spite of being considered as a psychogenic stress, restraint caused a stronger increase in liver MT levels than physical stresses previously used. In addition, it is noteworthy that the fold increase of MT was dependent on the method of quantitation. Thus, the numerous workers who use Zn on MT as a measure of MT level should be careful in interpreting the results so obtained.

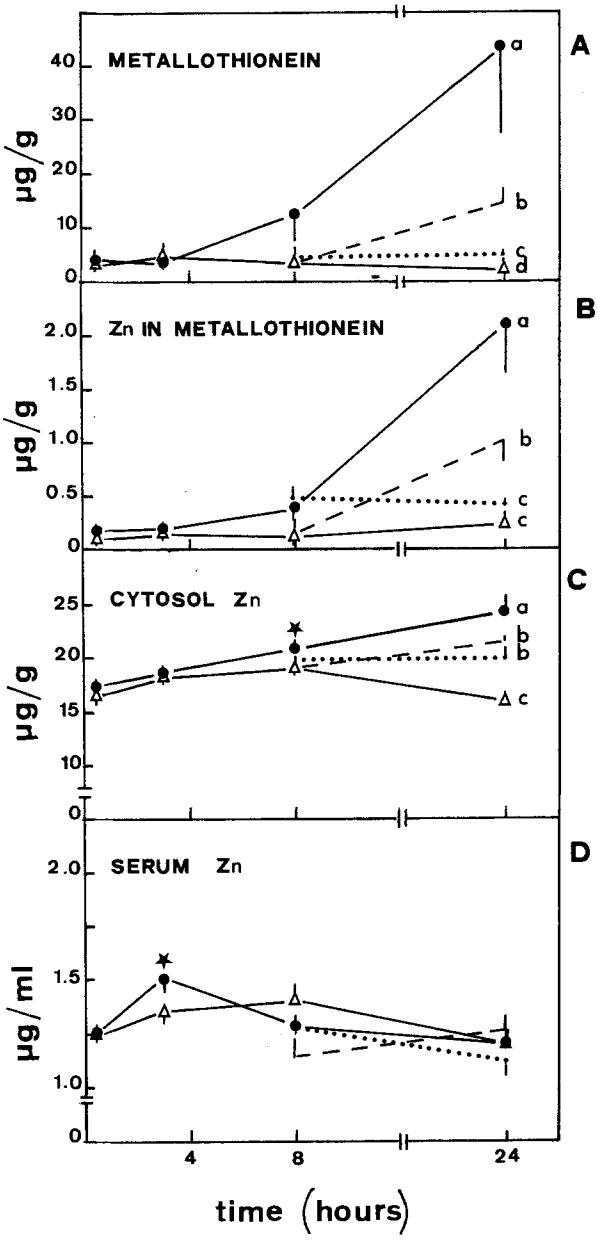

Figure 2. Effect of restraint and deprivation on A) metallothionein, B) Zn on metallothionein, C) cytosolic Zn and on D) serum Zn levels. The symbols are the means ± SE of at least 5 rats. (△—△), control; (●—●), restraint; (...), restraint+resting; (---), deprivation. For statistical analyses see text. *$p < 0.05$ versus control or control and the other two groups. Groups labeled with different letters are each statistically different from the other groups ($p < 0.05$).

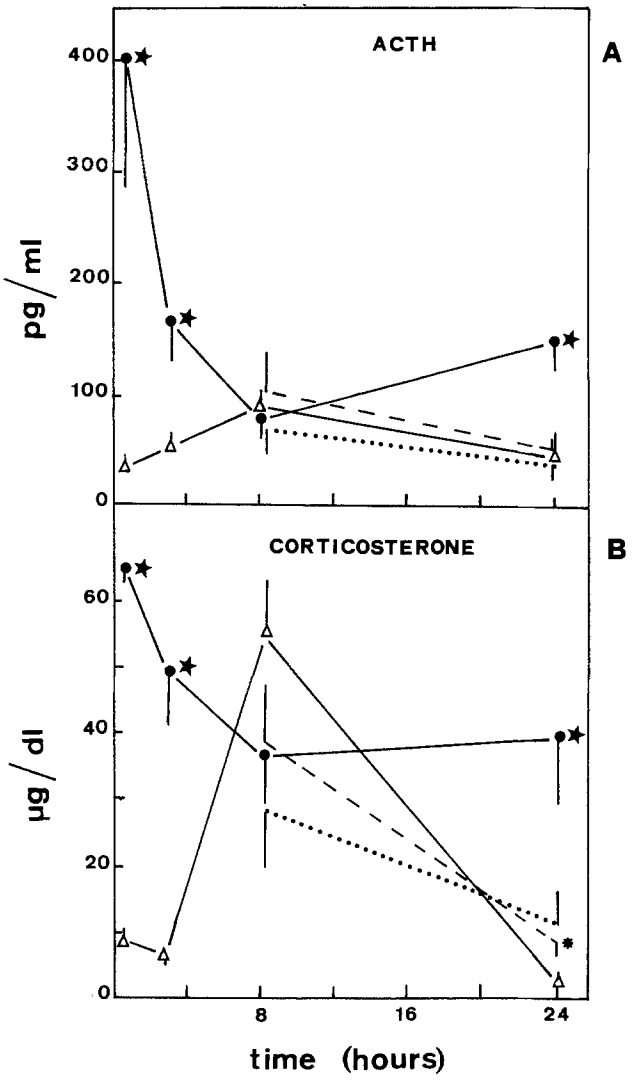

Figure 3. Effect of restraint and deprivation on serum (A) ACTH and (B) corticosterone. The symbols are the means ± SE of at least 5 rats. (△—△), control; (●—●), restraint; (...), restraint+resting; (---), deprivation. For statistical analyses see text. *A* *$p < 0.05$ versus control or control and the other two groups. *B* In 0.5- and 3-h groups, ★$p < 0.05$ versus the control group. In 24-h groups, ★$p < 0.05$ versus the other groups, and * $p < 0.05$ versus the control group.

Since the animals exposed to restraint cannot eat, an additional control group of animals deprived of food and water for the 8- and 24-h periods was used. The 24-h deprived animals showed a significant increase (598% as measured by MT RIA and a 423% as measured by Zn on MT) in liver MT, in accordance with a previous report[8]. Thus, we have been able to separate the effect of stress from that of food and water deprivation. This is particularly interesting, since the earlier studies[3,5,6,10] did not control the effect of the stress stimuli used on food intake, although it was very likely that the stress altered water and food consumption[12]. Rats exposed to 3 h of restraint and then returned to their home cages in the animal room showed higher hepatic MT levels than control rats after 21 h of resting (218% as measured by MT RIA and 180% as measured by Zn in MT). This suggests that metabolic or hormonal factors triggered by the initial exposure to restraint were sufficient to induce a slight increase in hepatic MT after a latency period necessary to induce the synthesis of this protein. The fact that 24 h of restraint produced a stronger increase of liver MT indicates that the effect of stress on hepatic MT is not qualitative but is clearly dependent on the duration of

the stress. Whether or not liver MT values are sensitive to the intensity of stress remains to be established.

Liver cytosolic and serum Zn levels are depicted in figures 2C and 2D, respectively. Three h of restraint induced a transient increase in serum Zn levels ($p < 0.05$) with return to control values after 8 h of restraint. In spite of there being no change in serum Zn, 24 h after onset of stress all the treated groups (stressed, stressed plus resting, deprived) showed higher liver cytosolic Zn than control rats ($p < 0.05$); restrained rats had the highest Zn levels. However, no conclusions can be drawn about whether or not there has been a reorganization of the liver Zn stores or whether some additional Zn has been introduced into the liver, since although serum Zn did not change, different Zn mobilization rates may have been present. On the other hand, fasted rats showed unaltered serum Zn levels, in agreement with a previous report[8], whereas control rats showed the expected serum Zn circadian rhythmicity[36].

The table shows serum MT values in all the experimental groups. Despite the significant changes in liver MT content, serum MT was not modified significantly by any experimental procedure. The fact that serum MT levels remained relatively unchanged was not unexpected. It was previously observed[37] that a single i.p. injection of Zn at a sublethal level (5 mg Zn/kg b. wt) produced a MT response in serum that was only slightly above control levels even 3 days after injection. A second injection at 3 days was required to produce a significant increase in serum MT levels (a factor of 2 above control levels). It is not unexpected that a less severe challenge to MT homeostasis, produced by stress, would produce a MT response in serum after 24 h but little changed from control levels. The mentioned observation[37] demonstrated that the response to one injection of 0.8 mg Cd/kg b. wt was more immediate; serum MT levels increased to 4 times control levels after 24 h and to 10 times control levels after another 24 h. However, a recent report[8] has demonstrated that endotoxin or CCl_4 administration caused not only liver MT induction but a strong rise in serum MT as well. These authors suggested that this increase in serum MT would be due to the liver damage produced by those stimuli. This hypothesis is supported by our results, since restraint probably did not cause liver damage and no change in serum MT was found after 24 h of restraint stress. Particularly noteworthy was the high variability in serum MT values (table) in all the groups. It seems possible that the estrous period has some influence on serum MT levels; this requires further investigation.

Figure 3 shows serum ACTH and corticosterone levels. As expected, restraint induced a potent secretion of both hormones[38]. Two-way ANOVA revealed a significant effect of stress for both ACTH and corticosterone ($p < 0.001$), whereas the period of stress had a significant effect only for corticosterone ($p < 0.001$). Stress-induced hormone release was clearly reduced after the first 3 h of stress, and circulating corticosterone was similar in stressed and in control rats after 8 h of restraint. However, ACTH and corticosterone levels were again higher the next morning, as revealed by one-way ANOVA and the additional Duncan procedure ($p < 0.05$). Probably, continuous corticosterone release during the morning could alter subsequent corticosterone release through corticosterone feedback on the hypothalamus-pituitary-adrenal axis[39], since an interaction between prolonged stress and circadian rhythmicity has been suggested[40]. Rats subjected to 24 h of water and food deprivation showed higher corticosterone but not higher ACTH levels than control animals. This could be due to the fact that reduced food intake also diminished hepatic corticosterone metabolism[12], leading to increased serum corticosterone without enhanced corticosterone release.

Glucocorticoids have been related to MT regulation by in vivo and in vitro studies[20-26]. However, comparison of pituitary-adrenal hormone levels and liver MT suggests that there is no clear relationship between serum corticosterone and liver MT. Firstly, a clear circadian rhythmicity of serum corticosterone was observed in accordance with previous reports[36]. However, no sign of circadian rhythmicity in liver or serum MT was found. Serum corticosterone levels were higher in the evening than in the morning in control rats. Therefore, it would be expected that liver MT levels would be elevated in the early morning, taking into consideration the delay in glucocorticoid induction of MT[24], and the half-life of Zn MT (about 19 h)[1]; this did not, however, occur. Secondly, adrenalectomy did not prevent liver MT induction by cadmium, turpentine[27] and interleukin-1[41]. Thirdly, corticosterone administration did not increase liver MT[3]. Thus, it seems that MT induction by stress must be regulated at least partially by mechanisms other than those related to glucocorticoids. In this regard, it is noteworthy that glucocorticoids, heavy metals and inflammatory agents induce MT1-mRNA accumulation by independent mechanisms[42]. Whether or not these or other mechanisms are implicated in MT induction by stress remains to be established. At present, catecholamines remain as the most important system in MT regulation during stress, since it has been demonstrated that a catecholamine blockade diminishes MT induction by surgery[3].

Serum levels of metallothionein in all the experimental groups

Hours	Control	Restraint	Restraint + resting	Deprivation
0.5	5.03 ± 0.91	5.04 ± 1.51	–	–
3.0	3.74 ± 1.60	3.05 ± 0.71	–	–
8.0	4.85 ± 0.72	4.66 ± 0.63	4.13 ± 1.18	5.36 ± 1.18
24.0	9.48 ± 4.80	8.56 ± 3.52	7.82 ± 2.39	6.00 ± 1.65

Serum levels of MT (ng/ml) in control, restraint, restraint + resting and water + food deprived rats after onset of stress (0.5, 3, 8, and 24 h). Results are mean ± SE from at least 5 rats, except 8 h restrained and deprived rats, where n = 3.

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On the renal inner medullary concentration of sodium

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19 November 1985

Summary. The principle of 'central volume' is applied to the sodium and water contents of the inner medulla of the mammalian kidney. The analysis raises questions about the possibility of concentrating sodium ions in the inner medulla by a mechanism that postulates, in the same tissue segment, water withdrawal from the descending thin limb of the loop of Henle and sodium entry from the ascending thin limb.
Key words. Central volume principle; kinetics of water and sodium; transit times of water and sodium; transport of water; water withdrawal.

The purpose of this paper is to re-examine the inner medullary concentrating process in the mammalian kidney, from a point of view heretofore not considered, in order to point out some problem which seem as yet unresolved.

Solute concentration in the medullary interstitium is higher than that in the systemic blood plasma: e.g. the concentration of Na is approximately twice as high in the inner medulla as in plasma. Hypotheses have been proposed postulating mechanisms of countercurrent solute recycling either in an active[1–3] or in a passive[4–6] mode, and of water extraction from descending channels[7]. Nevertheless, the mechanisms which generate high solute concentrations in the inner medulla are as yet not fully understood. To evaluate the potential mechanisms involved in the generation of high Na concentration increasing towards the papilla, it is helpful to look at the kinetics of solute and water movements across the medullary tissue in steady state conditions. For this purpose, divide the inner medulla into segments with imaginary surfaces parallel with the corticomedullary (C–M) border (i.e. perpedicular to the direction of the tubular and vascular loops and collecting ducts). Number the segments serially, from 1 to n, with the first nearest to the C–M border. Denote the amounts of extracellular Na and water present in the ith segment (including those in vascular and tubular channels) as $m_{i,s}$ and $m_{i,w}$, respectively, $i = 1,2,\ldots n$. Thus the average extracellular concentration of Na in the ith segment is $\frac{m_{i,s}}{m_{i,w}}$.

Let $f_{i,s}$ and $f_{i,w}$ be the flows of Na and water through the entire ith segment and let $t_{i,s}$ and $t_{i,w}$ be the mean transit or residence times of Na and water, respectively, in the ith segment. Sodium or water flows represent total flows occurring by convection, active transport, and diffusion, and are the sums of both descending and ascending flows entering the particular segment. The driving force for all these flows is the blood pressure, i.e. the pressure which drives the descending vascular and tubular flows into the medulla. Mean transit times represent weighted average transit times by all means of transport.

Since both sodium and water are in dynamic steady state in each segment, i.e. the inflows of sodium and water into each segment are equal to their respective outflows from that segment, the central volume principle is applicable: $m_{i,s} = f_{i,s} \cdot t_{i,s}$, i.e. the amount of sodium in segment i can be expressed as the product of flow of sodium through the segment and the mean transit time of sodium in that segment.

Thus we write

$$\frac{m_{i,s}}{m_{i,w}} = \frac{f_{i,s} \cdot t_{i,s}}{f_{i,w} \cdot t_{i,w}}.$$

If in the (i + l)st segment (nearer to the papilla than is the ith) the concentration of Na is higher than in the ith, i.e.

$$\frac{m_{i+1,s}}{m_{i+1,w}} > \frac{m_{i,s}}{m_{i,w}},$$

then

$$\frac{f_{i+1,s} \cdot t_{i+1,s}}{f_{i+1,w} \cdot t_{i+1,w}} > \frac{f_{i,s} \cdot t_{i,s}}{f_{i,w} \cdot t_{i,w}}.$$

The following table lists four propositions which, if true, would contribute to increasing Na concentration with depth in the medulla. The table also shows our current state of knowledge of each proposition.

Propositions	Inner medulla
1. $f_{i+1,s} > f_{i,s}$	False
2. $t_{i+1,s} > t_{i,s}$	?
3. $f_{i+1,w} < f_{i,w}$	True
4. $t_{i+1,w} < t_{i,w}$	?

To evaluate the truth of propositions 1 and 3, we have assumed that there is no entry of Na and water from the pelvic urine. Then, in steady state the amount of either Na or water entering