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THIOSULFATE ACCUMULATION BY RAT RENAL CORTEX SLICES

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SUMMARY

$S_2O_3^{2-}$ excretion in the dog has been well documented, and its sensitivity to certain hormones has been reported. For example, pretreatment of the female dog with cortisone promotes renal tubular secretion of $S_2O_3^{2-}$. This study was designed to establish the parameters for $S_2O_3^{2-}$ transport by isolated rat renal tissue prior to investigating the hormone effects. Standard renal slice procedures were employed with incubations performed in modified Krebs–Ringer phosphate solutions. Rat cortex slices accumulate $^{35}SSO_3^{2-}$ to steady-state distribution ratios (concentration in cell water/concentration in bathing solution) of about 3.0 in 90–120 min. Steady-state distribution ratios were depressed as the concentration of $S_2O_3^{2-}$ in the bathing solution was increased. No metabolic substrates were found to increase accumulation. Specifically, glucose had no effect, while a large number of organic acid intermediates (*e.g.* acetate, succinate) reduced accumulation. The uptake was energy dependent, *i.e.* it was reduced by metabolic inhibitors. $S_2O_3^{2-}$ accumulation was influenced by the K^+ concentration of the bathing solution, and perhaps to a slight extent by the Na^+ concentration. Specific activity measurements and high-voltage electrophoresis analyses indicated that $^{35}SSO_3^{2-}$ was neither metabolized by this slice system nor bound significantly to macromolecules. However, a small amount of trichloroacetic acid-insoluble label was detected.

INTRODUCTION

Interest in the biological distribution of $S_2O_3^{2-}$ started at the time of World War II when this substance was being investigated for its antidotal properties. From these studies the conclusion was reached that $S_2O_3^{2-}$ was excreted by glomerular filtration[1]. Extensions of these studies and others in man tended to confirm the original observations and led to the suggestion that $S_2O_3^{2-}$ clearance could be used as a measure of glomerular filtration rate[2,3,10].

Subsequently, however, the observation was made that pretreatment of the female dog with large doses of cortisone caused the $S_2O_3^{2-}$-to-creatinine clearance ratio to become greater than one. This was taken to mean that the hormone treatment enhanced or initiated renal tubular secretion to $S_2O_3^{2-}$ (ref. 4). That clear-cut secretion of $S_2O_3^{2-}$ could occur was documented in the case of the cat which routinely secreted

this ion[5]. More recent studies by BERGLUND *et al.*[6] and by MUDGE *et al.*[7] have attempted to clarify the physiological mechanisms involved in the renal handling of $S_2O_3^{2-}$.

The studies of MUDGE *et al.*[7] using the stop-flow technic showed that both secretory and reabsorptive activity were possible in the female dog. Proximal tubular secretory activity was initiated or enhanced by cortisone pretreatment. A marked reabsorptive capacity could be demonstrated in the same experiments where secretion was found, so it is probable that the hormone increased secretion rather than reduced reabsorption. In order to better study this hormone-stimulated secretory process it was decided to establish the parameters for $S_2O_3^{2-}$ transport in an *in vitro* system. The present investigation describe some of the characteristics of $S_2O_3^{2-}$ uptake by rat renal cortex slices. It is anticipated that these characteristics can be monitored in an evaluation of the hormone effects on the transport process.

METHODS

Each experiment was performed on the renal cortex tissue pooled from four to eight Sprague–Dawley rats. Rats of either sex were used and in general these animals weighed from 190 to 240 g. The animals were sacrificed by a blow to the base of the skull and the kidneys removed immediately and placed in cold Krebs–Ringer phosphate solution. Each kidney was cut longitudinally into two halves and each half in turn was cut into two cubes. Free-hand slices of the cortex were cut from each cube of kidney and these were stored in cold Krebs Ringer phosphate until used. The experiments were started routinely within 45 min of sacrificing the rats.

The incubations were performed either in a New Brunswick Metabolyte or a Dubnoff metabolic shaker. The shaking speed was between 80 and 90 cycles per min. Unless otherwise specified the bathing solution used for the incubations was a Krebs–Ringer phosphate solution containing 1 mM Ca^{2+} and 5 mM K^+ and was used in a volume of 3.0 ml per beaker. The gas phase utilized was 100% O_2, and the temperature 25°. Accumulation of $S_2O_3^{2-}$ by the slices was monitored using $Na_2{}^{35}S_2O_3$ (inner sulfur labeled, New England Nuclear Corp.). In general, enough unlabeled $Na_2S_2O_3$ was added to the incubation media to give a total chemical concentration of about 10 μM. The isotope concentration was 0.02 μC/ml.

At the end of the experimental period, the tissue slices were blotted on moist filter paper, weighed quickly and placed in distilled water prior to homogenization. After grinding, dilution and precipitation of proteins with trichloroacetic acid (final concn. of 5%), the samples were centrifuged. Aliquots of the supernatants were mixed with BRAY'S solution[8] and counted in a liquid scintillation spectrometer equipped with an external standard for quench correction. The incubation media were processed similarly. Total recoveries of radioactive label were calculated routinely and varied from 88 to 100%. In those few experiments where the radioactivity of the trichloroacetic acid precipitates were counted, these were washed as described previously[9], and dissolved in Soluene-100 (Packard Instrument Co.). Aliquots of these preparations were also counted by liquid scintillation technics.

In the experiments where electrophoresis was performed, the tissues were homogenized as usual, but trichloroacetic acid was not added. After centrifugation the "supernatant" was spotted on Whatman 3MM chromatography paper along with appropriate standards. The electrophoresis was performed on a Savant high-

voltage electrophoresis flat plate for 45 min at about 75 V/inch with a barbital buffer (pH 8.4). Aliquots of incubation media were prepared similarly for electrophoresis. Appropriate standards were run along with the tissue extracts as well as with the incubation media.

For the specific activity measurements, chemical determinations were done using the "indirect" method of NEWMAN *et al.*[10] on aliquots of tissue extracts that were also counted for radioactivity. All of the chemical determinations were corrected for tissue blanks determined on samples incubated under conditions identical with those for the experimental samples except for the absence of added $S_2O_3^{2-}$. The data are expressed as a ratio of the tissue specific activity (counts/min per μg) to the medium specific activity.

The accumulation data are expressed as distribution ratios. These were calculated as the ratios of radioactivity in cell water to that bathing solution. In experiments parallel to those in which $S_2O_3^{2-}$ uptake was measured, total tissue water was determined to be the difference in tissue weight before and after drying at 100° for 24 h. The extracellular component of the tissue water was determined with [^{14}C]-inulin (New England Nuclear Corp.). Details of such procedures have been presented by many investigators, *e.g.* KLEINZELLER *et al.*[11] and ROSENBERG *et al.*[12].

When necessary statistical calculations were employed using Student's *t* test. In every case where these analyses were performed the *N* given refers to the number of experiments.

RESULTS

Time-course of label uptake

The time-course of accumulation of radioactive sulfur (^{35}S) is given in Fig. 1. This uptake curve was in no way remarkable and indicated that label uptake had reached a steady state by 90–120 min. In all subsequent experiments 2-h accumulation studies were performed so as to insure that the measurements were made in the steady state.

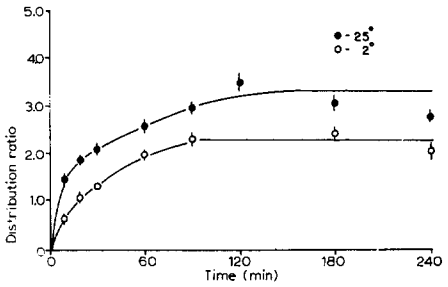


Fig. 1. Time-course of accumulation for $^{35}SSO_3^{2-}$ at each of two temperatures. The points are the means of from four to six experiments and the vertical lines are the standard errors.

Fate of label in tissue

Three approaches to this question were undertaken. First, for every experiment the total recovery of label was calculated routinely. At the end of the incubation the radioactivity in the tissue and that in the bathing solution were summed and

compared to that in control incubations, *i.e.* no tissue present. The radioactive material recovered ranged from 87 to 106% of control with a mean value of 96%. This indicated that virtually all of the label was present in the incubation media and tissue extracts. However, for completeness the trichloroacetic acid-insoluble residue was also examined, and these data are in Table I. Incubation for 2 h in a bathing solution containing 5 mM K^+ showed that a small, but significant, amount of label (2% of total) was retained in the trichloroacetic acid-insoluble tissue material. Interestingly if the incubation was in the presence of 40 mM K^+ the percent of

TABLE I

INCORPORATION OF $^{35}SSO_3^{2-}$ IN TRICHLOROACETIC ACID-INSOLUBLE FRACTION OF TISSUE HOMOGENATES

Details are presented in the text.

	% of total label in trichloroacetic acid precipitate	
After incubation in 5 mM K^+	2.02 ± 0.11	$P<0.01$
After incubation in 40 mM K^+	0.77 ± 0.14	

TABLE II

HIGH-VOLTAGE ELECTROPHORETIC MIGRATION OF ^{35}S ISOLATED FROM KIDNEY CORTEX SLICES AND THE BATHING MEDIA

The electrophoresis was performed at 2500 V in a 0.05 M barbital buffer (pH 8.4) for 45 min. Each value is the mean of triplicate analyses on each of three samples.

	Distance from origin toward +pole (cm)
$^{35}SSO_3^{2-}$ in water	14.7
Tissues extracted in water	15.0
$^{35}SSO_3^{2-}$ in Krebs–Ringer phosphate	6.0
Media samples after incubation	5.9

trichloroacetic acid-insoluble label was reduced significantly to less than 1% of the total.

Secondly, to examine the possibility that the $^{35}SSO_3^{2-}$ might be bound to some macromolecular structure, electrophoresis of tissue homogenates was performed and the data are presented in Table II. No alteration in the migration of the label was detected for either the tissue extracts or the bathing solution. If the labels had been bound to a macromolecule the migration would certainly have been different than that for the simultaneously run standard.

Some workers have suggested that a modest degree of $S_2O_3^{2-}$ oxidation to SO_4^{2-} can occur in rat kidney tissue[13]. To investigate this possibility in the present experimental system the $S_2O_3^{2-}$ specific activity was examined and the data are presented in Table III as the ratios of the tissue specific activity to the control bathing solution specific activity. In every case this ratio approximated 1.0. This was true

TABLE III

$^{35}SSO_3^{2-}$ SPECIFIC ACTIVITY RATIOS

Incubations were for 2 h at 25°. See text for complete explanation.

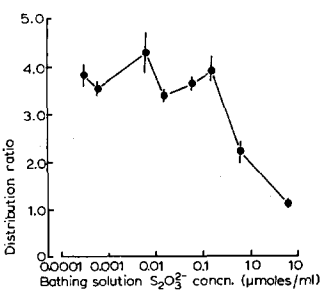
Bathing solution	$S_2O_3^{2-}$ *concn.* (*μg/ml*)	*Tissue specific activity* / *Medium specific activity*
5 mM K^+	100	1.035
	1000	1.195
40 mM K^+	10	0.860
	100	0.835
	1000	1.19

whether the bathing medium K^+ concentration was 5 or 40 mM. Further, the specific activity ratio was unaffected by the concentration of $S_2O_3^{2-}$ in the bathing solution.

In as much as (a) the label added at the start of the incubation was nearly fully recoverable, (b) the electrophoretic migrations of the label in aqueous extracts of the tissue and in the bathing solution were the same as for appropriate standards, and (c) the specific activity measurements did not indicate $S_2O_3^{2-}$ oxidation, it is reasonable to conclude that the ^{35}S exists in the tissue as authentic $^{35}SSO_3^{2-}$. That is, the labeled material was not incorporated chemically into the protein structure, it was not accumulated by non-specific binding, and it was not converted to the chemical relative, SO_4^{2-}.

Effect of $S_2O_3^{2-}$ concentration

In Fig. 2 the $S_2O_3^{2-}$ distribution ratios are plotted against different bathing solution concentrations of $Na_2S_2O_3$. The accumulation of this ion was insensitive to elevations in the $S_2O_3^{2-}$ level at low $S_2O_3^{2-}$ concentrations. Only above concentrations of 0.1 mM did apparent saturation of the accumulation process occur as evidenced by a significant reduction in the distribution ratio. Most of the subsequent studies reported here were conducted at about 0.1 mM well below the saturation concentration.



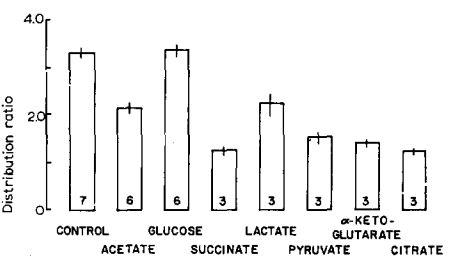


Fig. 2. The effect of the bathing solution $S_2O_3^{2-}$ concentration on the uptake of $^{35}SSO_3^{2-}$ by rat renal cortex slices. Points are means of from three to six experiments and the vertical lines are standard errors .

Fig. 3. The effect of potential metabolic substrates on $^{35}SSO_3^{2-}$ distribution ratio. Each bar represents the mean and each vertical line the standard error for the number of experiments indicated below the bar. The substrates were tested in a concentration of 0.01 M.

TABLE IV

EFFECTS OF METABOLIC INHIBITORS ON $^{35}SSO_3^{2-}$ DISTRIBUTION RATIO

The P values were determined relative to the control.

Inhibitor	*Concn.* (μM)	*N*	*Distribution ratio* $\pm$ *S.E.*	*P*
Control		8	3.55 $\pm$ 0.12	—
2,4-Dinitrophenol	50	5	1.13 $\pm$ 0.06	$<$0.01
	100	7	1.17 $\pm$ 0.05	$<$0.01
Iodoacetamide	100	3	1.55 $\pm$ 0.10	$<$0.01

Effects of metabolic inhibitors and substrates

2,4-Dinitrophenol, iodoacetamide (Table IV) and incubation in a N_2 atmosphere (data not presented) were examined for effects on the $S_2O_3^{2-}$ distribution ratio. All reduced significantly the accumulation of the label.

Various substances thought to serve as substrates for renal metabolic processes or at least capable of modifying certain renal transport processes were examined for their effects on $S_2O_3^{2-}$ uptake (Fig. 3). No substrate was found to enhance $S_2O_3^{2-}$ accumulation, and only glucose was found to be without effect. All the other substances depressed significantly the distribution ratio. Acetate and lactate produced less depression than did the other organic acids tested, however.

Effects of anions

Two inorganic anions were tested for their effects on $S_2O_3^{2-}$ uptake (Table V). Both SO_4^{2-} and NO_3^- were examined over a wide concentration ranges (0.01–10 mM). Only SO_4^{2-} significantly reduced the $S_2O_3^{2-}$ distribution ratio and then only when present in the highest concentration tested (0.01 M).

Two organic anions, carinamide and probenecid, were also tested in this

TABLE V

EFFECTS OF INORGANIC ANIONS ON $^{35}SSO_3^{2-}$ DISTRIBUTION RATIO

The P value was determined relative to the control.

	Concn. (μM)	*N*	*Distribution ratio* $\pm$ *S.E.*	*P*
Control	—	4	3.53 $\pm$ 0.30	
Na_2SO_4	0.01	4	3.08 $\pm$ 0.15	—
	0.1	4	3.28 $\pm$ 0.32	—
	1.0	4	3.27 $\pm$ 0.21	—
	10	4	1.48 $\pm$ 0.11	$<$0.01
$NaNO_3$	0.01	4	3.36 $\pm$ 0.21	—
	0.1	3	3.50 $\pm$ 0.16	—
	1.0	4	3.47 $\pm$ 0.18	—
	10	4	3.13 $\pm$ 0.17	—

TABLE VI

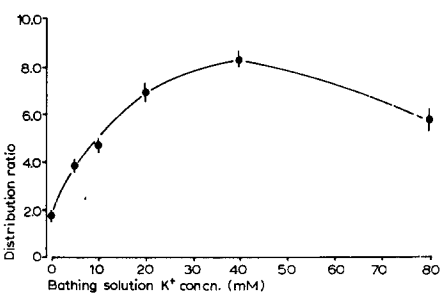
EFFECTS OF TWO ORGANIC ANIONS ON $^{35}SSO_3^{2-}$ DISTRIBUTION RATIOS

	Concn. (μM)	*N*	*Distribution ratio* ± *S.E.*
Control	—	4	3.95 ± 0.20
Carinamide	1	4	3.79 ± 0.16
	10	4	3.95 ± 0.09
	100	4	3.52 ± 0.17
Probenecid	1	4	3.83 ± 0.12
	10	4	3.73 ± 0.08
	100	4	3.34 ± 0.11

system. Carinamide and probenecid are chemically closely related, but only carinamide significantly reduced $S_2O_3^{2-}$ secretion by the intact dog[7]. In this *in vitro* system neither compound altered the $S_2O_3^{2-}$ distribution ratio.

Effects of cations

Certain renal transport processes are known to be influenced by the bathing solution cation composition[14–16]. The effects of K^+ and Na^+ were examined, therefore, for their effects on $S_2O_3^{2-}$ uptake. In Fig. 4 the distribution ratio for $^{35}SSO_3^{2-}$ is plotted against the bathing solution K^+ concentration. For these experiments the



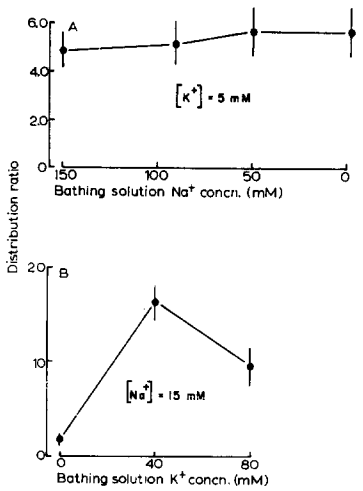


Fig. 4. Effect of increasing the K^+ concentration on $^{35}SSO_3^{2-}$ distribution ratio. Each point is the mean of four experiments and the vertical line the standard error. Osmolality was maintained by reductions in the bathing solution concentration of Na^+.

Fig. 5. A. Effect of reducing the Na^+ concentration of the bathing medium in the presence of a constant K^+ concentration on the $^{35}SSO_3^{2-}$ distribution ratio. Each point is the mean of four experiments and the vertical line is the standard error. Osmolality was maintained with sucrose. B. Effect of K^+ on $^{35}SSO_3^{2-}$ distribution ratio. The Na^+ concentration was 15 mM in all three experimental situations with sucrose added as needed to maintain the osmolality at a physiological level. Each point is the mean of four experiments and the vertical line is the standard error.

osmolality was maintained constant by reducing the Na^+ concentration as the K^+ concentration was elevated. The lowest distribution ratio was obtained in the absence of K^+. $S_2O_3^{2-}$ uptake increased as the K^+ concentration was increased with an apparent maximal accumulation at 40 mM K^+.

In an attempt to separate the effect of increasing the K^+ from reducing the Na^+ concentration of the bathing solution two additional sets of experiments were performed. In the top panel of Fig. 5, the medium K^+ concentration was kept constant at 5 mM while the medium Na^+ concentration was reduced from about 150 to about 15 mM. Osmolality was maintained with sucrose. No effect was noted on $S_2O_3^{2-}$ uptake as the Na^+ concentration was reduced. In the bottom panel of Fig. 5, the Na^+ in the bathing solution was maintained at 15 mM. In the absence of K^+ relatively little accumulation was noted, while at 40 mM K^+ the distribution ratio was over 16.0.

DISCUSSION

Taken together these data are consistent with an active accumulation of $S_2O_3^{2-}$ isolated rat renal cortex slices. The studies on the fate of the label in the tissue indicated that it existed as free, authentic $S_2O_3^{2-}$ and not in a bound form or as a metabolite. Although nothing specific is known of the electrical gradient in these isolated renal slices, it is likely that the cellular potentials were negative with respect to the extracellular spaces. Such a gradient would not facilitate $S_2O_3^{2-}$ movement into the cells. But in fact the distribution of the label was from 3 to 16 times as great in cell water as in the bathing solution (depending on experimental conditions), which indicates that the label must have been taken up against its concentration gradient. In addition the reduction in uptake caused by elevating the bathing solution $S_2O_3^{2-}$ concentration is consistent with a carrier-mediated accumulation process. Finally, the reduction in uptake produced by metabolic inhibitors is what would be expected if one is dealing with an active transport system requiring energy expenditure by the tissue.

SO_4^{2-} and NO_3^- were examined for their effects on $S_2O_3^{2-}$ uptake in an attempt to learn about the anion specificity of this transport process. The details of the $S_2O_3^{2-}$–SO_4^{2-} interaction in this *in vitro* system will be dealt with in a subsequent paper, but it is clear from this study that although SO_4^{2-} blocked $S_2O_3^{2-}$ uptake the effect was not marked. No effect was seen even at a SO_4^{2-}:$S_2O_3^{2-}$ concentration ratio of 100. Not until the SO_4^{2-} was present in 1000 times the $S_2O_3^{2-}$ concentration was an effect seen. Incidentally this is about the same order of inhibition as noted by DEYRUP[17] in her studies on the effect of $S_2O_3^{2-}$ on SO_4^{2-} uptake. In those experiments the SO_4^{2-} concentration was 0.5 μM and a significant depression of uptake was not seen until the $S_2O_3^{2-}$ concentration was raised to 0.1 mM.

The relationship of carinamide and probenecid to renal $S_2O_3^{2-}$ transport *in vivo* has been described[9]. In the dog that was capable of $S_2O_3^{2-}$ secretion as jugded by stop-flow analysis, carinamide significantly reduced the secretory activity. Probenecid, however, failed to exert any effect in the stop-flow studies. If for $S_2O_3^{2-}$ one can equate *in vitro* accumulation to *in vivo* secretion, as is generally accepted for organic acids such as *p*-aminohippurate, then the failure of carinamide to have an effect in this *in vitro* system may indicate that $S_2O_3^{2-}$ is handled differently in

the rat than the dog. On the other hand, this negative result may indicate that carinamide failed to penetrate to its site of action in the tissue slice system whereas in the intact dog after intravenous administration it more readily reached its site. Other organic anions, however, were capable of reaching a site of action as was demonstrated by the inhibition produced by acetate, lactate, *etc.* The mechanism by which these substances reduced the $S_2O_3^{2-}$ distribution ratio is entirely unknown.

It was interesting to note that $S_2O_3^{2-}$ uptake by rat renal slices shows a requirement for K^+ not unlike that noted for other substances[14,16,18]. The uptake of at least two organic acids, *p*-aminohippurate and urate, as well as that of SO_4^{2-}, has been reported to be much greater in the presence of high K^+ concentrations than in its absence. In the case of SO_4^{2-} (ref. 14) and $S_2O_3^{2-}$, however, the situation appears more complicated than with the organic acids. Reduction of the Na^+ concentration did not appear to influence $S_2O_3^{2-}$ uptake in the presence of low K^+. When the K^+ concentration was elevated to 40 mM, however, in the presence of low Na^+, a greater $S_2O_3^{2-}$ uptake was seen than when 40 mM K^+ was present along with 90 mM Na^+. This may indicate that some type of cation interaction is involved in the uptake process with Na^+ lack serving a permissive function rather than one of direct stimulation. No explanation is available at present for the role of K^+ in this transport system. Studies are in progress to examine tissue alkali cation concentrations and their relationship to $S_2O_3^{2-}$ uptake.

These experiments were designed to attempt a characterization of the rat renal cortex $S_2O_3^{2-}$ transport system. The following information relevant to this issue has been obtained: (a) $S_2O_3^{2-}$ is accumulated by slices of rat renal cortex against a concentration gradient by a specific, saturable, energy dependent process. (b) Some, but not all, of the organic acids tested reduced $S_2O_3^{2-}$ uptake. (c) This transport process, not unlike others, requires K^+ for maximal activity. The role of Na^+ is not clear.

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