

Extracellular Fluid in Individual Tissues and in Whole Animals: The Distribution of Radiosulfate and Radiobromide

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ABSTRACT Radiosulfate, $^{35}\text{SO}_4$, and radiobromide, ^{82}Br , were administered simultaneously to rats and dogs. In rats, the apparent volume of distribution of ^{82}Br averaged 30% of body weight and was constant between 0.5 and 35 hr after injection. The apparent volume of distribution of $^{35}\text{SO}_4$, corrected for urinary loss, increased by 6% body weight/hr: the extrapolated volume at zero time was 88% of bromide space. Analysis of individual tissues and carcasses for ^{82}Br and inorganic $^{35}\text{SO}_4$ showed that equilibration of both isotopes in several organs and in the whole carcass was rapidly achieved within 1 to 2 hr: no further increase in measured spaces occurred in 24 hr. The carcass inorganic sulfate space was 92% \pm 2% of the bromide space in intact rats, and showed no increase with time. However, a progressively greater fraction of the injected $^{35}\text{SO}_4$ was not recovered, owing to metabolic alteration. In eviscerated rats, the inorganic sulfate space was a smaller and much more constant fraction (79.8% \pm 0.4%) of the bromide space, showing that at least 20% of body bromide (and hence chloride) is nonextracellular. The viscera chiefly responsible for the higher ratio of spaces in the intact animal were the liver, small bowel, and kidney. In the last two organs, excess inorganic $^{35}\text{SO}_4$ (beyond the bromide space) was attributable to trapped transcellular fluid in which sulfate had been concentrated more than chloride (or bromide). Excess sulfate in liver and cartilage could not be explained in this manner; the results suggest passive binding of sulfate, but could reflect active cell uptake in

these tissues. No excess sulfate was found in skin or tail. The implications of these observations with respect to the distribution of body chloride and the measurement of extracellular space are discussed. The extracellular volume of the rat is estimated to be 24% of body weight.

INTRODUCTION

A useful definition of extracellular fluid volume should achieve two purposes: it should permit intracellular volume to be calculated by difference from total water, and it should delineate the amount of water available to act as a solvent for extracellular solutes. These two definitions may differ if appreciable quantities of extracellular water are unable to act as solvent water. At least as far as small ions are concerned, this difference is probably minor, and in our present state of knowledge must be overlooked.

It is less obvious whether the transcellular fluids should be included. These fluids are available to a limited and varying extent to buffer changes in both the composition and volume of the extracellular fluid. Therefore it would seem desirable to measure a compartment which includes all of the transcellular fluids, as well as the extracellular space, and to correct for these values individually, as is done in measuring total body water.

None of the indicator dilution methods for measuring extracellular space in tissues or in whole animals is generally accepted because most of the evidence supporting their use has been highly indirect. Unless an indicator can be shown rapidly to reach a volume of distribution, either in the whole organism or in individual tissues, that does not subsequently increase with time, any assertions as to the volume that corresponds to the true extracellular space are necessarily tentative. Furthermore, only indicator molecules as small as representative

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Received for publication 25 March and in revised form 27 June 1968.

extracellular electrolytes can be confidently expected to reach all of the extracellular solvent water that is accessible to these ions.

Sulfate has several characteristics which recommend it as an extracellular indicator (1), but it is well known that radioactivity accumulates in many tissues of laboratory animals given inorganic radiosulfate (2). This uptake could represent incorporation of sulfate into organic forms, or accumulation of inorganic sulfate as such. The relative magnitude of these two processes has seldom been examined. In the present work this question was investigated by analysis of tissues of rats and dogs and whole bodies of rats. We found the distribution of inorganic radiosulfate, as such, to become constant quite rapidly; by comparison with radiobromide distribution, we infer that radiosulfate space corresponds to extracellular space in some tissues, but not in all, and overestimates total extracellular volume by inclusion of some transcellular fluids as well. Nevertheless, a larger fraction of body bromide (and hence chloride) is non-extracellular.

METHODS

Nonfasting male Sprague-Dawley rats weighing 250–400 g were divided into three groups. All were anaesthetized with 50 mg/kg pentobarbital sodium intraperitoneally. We used the 22 rats in group I to measure the apparent volumes of distribution of ^{82}Br and $^{35}\text{SO}_4$. We injected intraperitoneally a measured volume of solution¹ containing approximately 10 μc each of $\text{Na}_2^{35}\text{SO}_4$ and K^{82}Br . At time intervals up to 3 hr, we opened the abdomen, aspirated the urine from the bladder, rinsed out the bladder three times with 2 ml of 5 mM Na_2SO_4 , and diluted the urine and washings to a total volume of 100 ml with 5 mM Na_2SO_4 . The animal was then exsanguinated from the abdominal aorta, and the serum separated under oil.

We used the 34 rats in group II for analysis of individual tissues. In group IIA, comprising 21 rats, of whom two were nephrectomized, samples of the following tissues taken (n = number of rats): kidney ($n=6$), liver ($n=9$), stomach ($n=4$), jejunum ($n=7$), large bowel ($n=4$), skin ($n=3$), tail ($n=2$), and knee ($n=5$). We loaded five rats in group IIB with stable sulfate by injecting subcutaneously 100 ml/kg of warmed solution containing 90 mM Na_2SO_4 , 7 mM K_2SO_4 , 3 mM CaCl_2 , and 4 mM MgSO_4 , 1/2 hr before the intraperitoneal injection of isotopes. Samples of liver ($n=5$) and knee ($n=5$) were taken. We infused eight rats in group IIC with a solution containing mannitol, 10 g/100 ml; inulin, 2 g/100 ml; 90 mM Na_2SO_4 with $\text{Na}_2^{35}\text{SO}_4$, 5 $\mu\text{c}/\text{ml}$; 7 mM K_2SO_4 , 3 mM CaCl_2 , and 4 mM MgSO_4 , via the external jugular vein at rates between 2 and 10 ml/hour. These animals were killed 1.2–2 hr after the start of the infusion and samples of blood, urine, and kidney were taken.

There were 10 rats in group III, in whom we measured ^{82}Br and inorganic $^{35}\text{SO}_4$ in the whole carcass. Five of these rats (group IIIA) were given ^{82}Br and $^{35}\text{SO}_4$ intraperitoneally, and at 1/2–2 hr urine was aspirated, blood with-

¹ $\text{Na}_2^{35}\text{SO}_4$, 426 mc/mmmole, was obtained from New England Nuclear Corp., Boston, Mass. K^{82}Br , > 82 mc/mmmole, was obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

drawn, and the carcasses prepared for analysis. In another four rats, the abdominal viscera were removed before the carcasses were processed. Two of these rats (group IIIB) were killed at 1–2 hr. The other two (group IIIC) were nephrectomized, given carrier-free $\text{Na}_2^{35}\text{SO}_4$ and ^{82}Br intravenously, and allowed to recover. 24 hr later they were anaesthetized. Blood and urine were collected and the carcasses processed. The abdominal viscera from these four rats were weighed, and aliquots taken. These tissue data have been included in the results presented for group II. The last rat (IIID) was given intraperitoneally 100 ml/kg of warmed carrier sulfate solution, containing 90 mM Na_2SO_4 , 7 mM K_2SO_4 , 4 mM MgSO_4 , and 3 mM CaCl_2 64 min before nephrectomy and the injection of isotopes intravenously, and was killed 122 min after the operation. No residual peritoneal fluid was evident.

We anaesthetized two fasting mongrel female dogs with 30 ml/kg pentobarbital intravenously, injected a priming dose of K^{82}Br , and $\text{Na}_2^{35}\text{SO}_4$ intravenously, and then infused $\text{Na}_2^{35}\text{SO}_4$ in glucose, 5 g/100 ml. Four successive liver biopsies were taken from each dog and four biopsies from the small intestine of one dog.

Tissue processing. We removed adherent blood from the liver and spleen samples, stripped the capsule and perirenal fat off the kidneys, and wiped clean the mucosal surface of the intestinal samples. At the same time, samples of the mixed contents of the entire intestines of the rats were taken for analysis. Aliquots of about 1 g were weighed and immediately homogenized in 5 ml of cold 5 mM Na_2SO_4 or 5 ml cold ethanolamine buffer (0.2 M ethanolamine, 0.1 M HCl, pH 10.5) containing 5 mM Na_2SO_4 and centrifuged. Another aliquot of tissue was dried to constant weight at 110°, and the water content determined, so that the final homogenate solvent volume could be estimated.

The knee was isolated by transecting the femur and the tibia and removing as much muscle and connective tissue as possible. We removed the hair from skin samples with a thioglycollate depilatory and these tissues, as well as the tail, which are refractory to homogenization, were immediately frozen in liquid nitrogen, and handled in a manner similar to that described below for the carcasses.

The rat carcasses were shaved and depilated. We removed the gastrointestinal contents and, in the eviscerated group, excised the liver, spleen, kidneys, ureter, bladder, and gastrointestinal tract. The carcasses were frozen in liquid nitrogen and were pulverized at that temperature, using a lead weight and a steel cylinder. They were then freeze-dried. Fat was extracted with 20 volumes of ethyl ether overnight. We ground entire carcasses in a Wiley mill, equipped with a 40 μ screen. At this stage the powder is not homogeneous, and is not easy to mix. We therefore extracted the whole rat powder for 2 hr at 4°C with 20 volumes (w/v) of either 5 mM Na_2SO_4 or ethanolamine buffer containing 5 mM Na_2SO_4 . An aliquot of the extract was centrifuged, and stored for subsequent analysis at 4°C. We initially deproteinized serum as follows: 1 volume was mixed with 4 volumes of 5 mM Na_2SO_4 . 1 volume of ammonium succinate buffer (0.25 M succinic acid, 0.3 M NH_4OH , adjusted to pH 4.51 with HCl) was added, and the mixture heated at 100°C for 5 min and centrifuged (3). In later experiments, we used the following method: 1 volume of serum was mixed with 4 volumes of 0.1 mM Na_2SO_4 , 1 volume 20% (w/v) trichloroacetic acid (TCA) was added, and the mixture was stirred and centrifuged. Tissue and carcass extracts were deproteinized with 5 volumes of uranyl acetate solution, as suggested by Miller, Hlad, Levine, Holmes, and Elrick (4): 8.0 g/liter of uranium acetate $[\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot$

2H₂O], 10.0 g/liter sodium acetate (NaC₂H₃O₂·3H₂O), and 25 ml/liter glacial acetic acid. This reagent has a pH of 4.1. Some of the kidney samples, as well as the dog liver and jejunal samples, were deproteinized with ammonium succinate buffer as above.

We estimated ⁸²Br in a gamma scintillation counter equipped with a well. Deproteinized serum or undeproproteinized supernatants of tissue extracts were counted. At least 10,000 sample counts were accumulated, and appropriate corrections made for background and physical decay. When more than three half-lives had elapsed from the date of assay, we checked the radiochemical purity of the ⁸²Br by determining the concordance of the observed and expected decay. Completeness of the extraction of ⁸²Br from the carcass powder was verified by comparing the counts on the dry tissue with those in the homogenate supernatant. A recovery of 101% ±2% (sd, n=6) was found. Extraction of ⁸²Br was complete at 20 min. Excluding one experiment in which some of the carcass was known to have been lost, and the two nephrectomized rats who had vomited and defecated, 91% ±3% (sd, n=6) of the administered ⁸²Br could be accounted for. The remaining 9% undoubtedly represents loss during the pulverizing, freeze-drying, and milling processes.

Inorganic radi sulfate was isolated as the benzidine (4,4'-diaminodiphenyl) salt: 1 volume of deproteinized serum, tissue extract, diluted urine, or injected solution was mixed with 5 volumes of benzidine dihydrochloride, 2 g/100 ml in 60% (v/v) acetone in a centrifuge tube. This reagent was prepared freshly by dissolving benzidine dihydrochloride in 20 volumes of hot water, and carefully adding 30 volumes of acetone. Benzidine sulfate was precipitated overnight at 4°, and isolated by centrifugation for 15 min, decanting the supernatant, and allowing the tubes to drain inverted for 10 min. The precipitates were dried in a stream of air to remove traces of acetone (which caused marked quenching) and were transferred by solution, with washings, in a total volume of 5 ml of 12% (v/v) monoethanolamine in methanol. 10 ml of scintillation fluid (2,5-diphenyloxazole, 4 g/liter; 1,4-*p*-bis[2-(5-phenyloxazolyl)] benzene, 0.2 g/liter in toluene) was added, and the vials were mixed and counted on a liquid scintillation counter. At least 10,000 sample counts were obtained, and corrections for background made. All samples were at least 10 times background. Contamination by ⁸²Br trapped in the benzidine sulfate precipitate was checked by observing the constancy of counts; on the rare occasions when a significant decline was observed, a correction was made. A correction for ²³⁸U trapped in the precipitate was made by counting at two channels, and never exceeded 10% of the ⁸²S counts. We added internal ⁸²S standard to all vials, and made an appropriate correction if a significant difference in efficiency between samples was observed.

The efficiency of counting of ⁸²S in this system was about 80%. All the ⁸²S counts added to a 5 mM Na₂SO₄ solution were recovered in the benzidine sulfate precipitate. The coefficient of variation of replicate precipitates from Na₂⁸²SO₄ standard solution was 0.57% (n=12). Variations in the amount of carrier sulfate between 0.4 and 9.5 mM did not affect the recovery of ⁸²SO₄. Recovery of ⁸²SO₄ added to rat serum was 95% ±2% (sd, n=6) and from dog serum 96% ±1% (sd, n=8) using the succinate method of deproteinization. With the TCA deproteinization we found the mean recovery of ⁸²SO₄ to be 98% ±3% (sd, n=30) from rat serum and 98% ±3% (sd, n=11) from dog serum. No correction has been made for the slight loss of radi sulfate during the succinate method of deproteinization. Recoveries of ⁸²SO₄ from the carcass suspension were identical with either neutral or alkaline extraction and averaged 98% ±3%

(sd, n=8). We found that the amount of ⁸²SO₄ obtained from the carcass powder by neutral aqueous extraction reached a plateau in 1/2 hr, but increased by a further 12% on standing overnight at 4°C; we attributed this increase to enzymic breakdown of organic sulfates. In one rat (IIIA1), the carcass was extracted overnight, and therefore an approximate 12% correction has been made; this animal is noted in the figure.

We introduced the alkaline extraction method to minimize the chemical and enzymic breakdown of labile organic sulfates, but we subsequently found in liver samples that the two methods gave identical results. Using uranyl acetate deproteinization, we obtained the following recoveries for ⁸²SO₄: liver, 94% ±3% (sd, n=4); kidney, 94% ±3% (sd, n=4); gut, 99% (n=2); tail, 99% (n=1); skin, 96% (n=2); knee, 98% (n=2); spleen 100% ±5% (sd, n=4). Using succinate method of deproteinization, ⁸²SO₄ recoveries from kidney were 98% (n=1), from liver, 93% (n=2), and from jejunum, 99% (n=1). No correction has been made for the slight losses in some of these recoveries.

Alkali-stable inulin was measured in serum, urine, and kidney by the method of Walser, Davidson, and Orloff (5). Kidneys were homogenized in 5 volumes of 5 mM Na₂SO₄. This extract was then treated as serum. Recovery of inulin from serum was 100% ±6% (sd, n=15) and from kidney, 100% ±5% (sd, n=16). Serum and tissue blanks were always less than 5% of the experimental samples.

Calculations. Volumes of distribution were calculated as the amount of isotope in the body (the difference between the quantity injected and the amount recovered in the urine) divided by the serum radioactivity.

Tissue and carcass spaces were calculated as the measured tissue or carcass content of isotope divided by serum radioactivity. In both calculations, corrections were made for an assumed serum water content of 93% and for Donnan factors of 0.95 for bromide and 0.90 for sulfate.

In the carcass analyses, a correction was applied to both ⁸²SO₄ and ⁸²Br carcass values to allow for loss of tissue during processing. We assumed that the fraction, *F*, of injected counts not recovered in the summated ⁸²Br contents of the blood sample, gut contents, bladder urine, cage washings, and carcass represented loss of a portion of the carcass. This assumption is reasonable, since losses inevitably occurred during the many transfers involved in processing the carcasses. The fraction lost averaged 12.8%. The measured carcass counts, *C*, of both isotopes were multiplied by the fraction (C + *F*)/*C*, obtained from the ⁸²Br results, to obtain corrected carcass radioactivity. The recovery of ⁸²Br, using this corrected value, was of course 100%. The recovery of ⁸²SO₄ in inorganic form was invariably incomplete, owing to metabolic alteration. Metabolized sulfate was calculated as the difference between the corrected percentage recovery of ⁸²SO₄ and 100%.

RESULTS

Apparent volumes of distribution of ⁸²Br and ³⁵SO₄

The data obtained from the rats in group I are displayed in Fig. 1. No significant correlation was found between *V*⁸²Br and time: *V*⁸²Br (ml/100 g body weight) = 26.9 (±0.7) + 0.56 (±0.37)*t*. Here, *t* = time (hr) after injection of the isotope. Previous work (6) has also revealed rapid equilibration of bromide in the rat, follow-

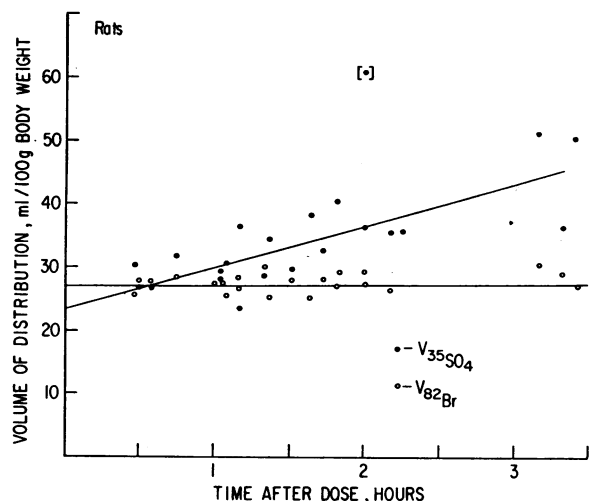


FIGURE 1 The apparent volumes of distribution of radio-sulfate and radiobromide in the rat. V is calculated as the quantity injected minus urinary excretion divided by the concentration in interstitial fluid. A single aberrant value of sulfate volume is enclosed by brackets and omitted from the calculation of the regression line. $V^{35\text{SO}_4}$ rises with time but $V^{82\text{Br}}$ does not. The ratio of $V^{35\text{SO}_4}$ to $V^{82\text{Br}}$ extrapolated to time of injection is 0.88.

ing parenteral administration, but most workers have not examined times as early as 1/2 hr.

In contrast, $V^{35\text{SO}_4}$ rose significantly with time: $V^{35\text{SO}_4}$ (ml/100 g body weight) = $23.6 (\pm 2.1) + 6.5 (\pm 1.2)t$. The rate of increase amounts to 6.5% of body weight per hr, considerably higher than that observed in man

(2.2%) or dogs (2.7%) (7). Whether the increase is actually linear cannot be stated with certainty. There is no apparent reason why it should be. However, it is sufficiently close to linear to employ linear regression for descriptive purposes.

When the relationship between $V^{35\text{SO}_4}/V^{82\text{Br}}$ in individual rats and time was examined, we found the extrapolated zero time estimate of $V^{35\text{SO}_4}/V^{82\text{Br}}$ to be 0.88: $V^{35\text{SO}_4}/V^{82\text{Br}} = 0.88 (\pm 0.08) + 0.22 (\pm 0.04)t$. The same value is obtained from the ratio of intercepts in the two preceding equations. If reverse extrapolation is valid, this value should give the ratio of body inorganic sulfate space to bromide space, independent of time. As shown below, this is approximately true.

Radiosulfate and radiobromide spaces of individual tissues

The results of rats in groups IIA and IIB and of the dogs are presented in Table I. $E^{35\text{SO}_4}$ exceeds $E^{82\text{Br}}$ in the liver, small bowel, kidney, and knee of the rat, and in the liver (but not the small bowel) of the dog. $E^{35\text{SO}_4}$ is less than $E^{82\text{Br}}$ in the stomach, large bowel, spleen, tail, and skin of the rat. Further study of these differences was made in liver, small bowel, kidney, and knee.

Liver. Radiosulfate rapidly penetrates beyond the radiobromide space of the rat liver to reach a value at 1–2 hr which is approximately 50% greater (Table I). Over the next 24 hr, no further increase occurs. In the rat, this excess sulfate cannot be attributed to contamination of the sample by bile, for hepatic bile has a higher concentration of ^{82}Br than $^{35}\text{SO}_4$ relative to serum (Ta-

TABLE I
Radiosulfate and Radiobromide Spaces of Tissues of the Rat and the Dog, Mean Values \pm SEM

Animals	Tissue and No. of samples	Radiobromide space ($E^{82\text{Br}}$)	Radiosulfate space ($E^{35\text{SO}_4}$)	Ratio of $E^{35\text{SO}_4}/E^{82\text{Br}}$	Relevant trans-cellular fluid	Fluid/serum $^{35}\text{SO}_4$ /Fluid/serum ^{82}Br
Rats, group IIA	Liver (9)	23 \pm 1*	36 \pm 1*	1.56 \pm 0.1	Bile (3)	0.53 \pm 0.06
	Stomach (4)	54 \pm 7*	28 \pm 2*	0.54 \pm 0.05	Gastric juice (4)	0.05 \pm 0.02
	Small bowel (7)	20 \pm 2*	42 \pm 8*	2.07 \pm 0.3	Contents (7)	3.7 \pm 0.8
	Large bowel (4)	28 \pm 2*	27 \pm 2*	0.92 \pm 0.1	Contents (4)	1.4 \pm 0.6
	Kidney (6)	50 \pm 1*	70 \pm 6*	1.50 \pm 0.2	Urine (6)	33 \pm 8
	Spleen (4)	41 \pm 1*	27 \pm 2*	0.67 \pm 0.04		
	Knee (5)	84 \pm 4†	98 \pm 6†	1.18 \pm 0.04		
	Tail (2)	89, 109†	81, 89†	0.91, 0.81		
	Skin (3)	224 \pm 14†	197 \pm 15†	0.88 \pm 0.04		
Rats, group IIB	Liver (5)	20 \pm 1*	30 \pm 2*	1.51 \pm 0.09		
	Knee (5)	77 \pm 4†	83 \pm 8†	1.11 \pm 0.18		
Dog 1	Liver (4)	23 \pm 1*	47 \pm 5*	2.06 \pm 0.25		
	Small bowel (4)	30 \pm 2*	27 \pm 1*	0.92 \pm 0.02		
Dog 2	Liver (4)	18 \pm 2*	27 \pm 2*	1.59 \pm 0.25		

* ml/100 gm wet weight.

† ml/100 gm fat-free dry weight.

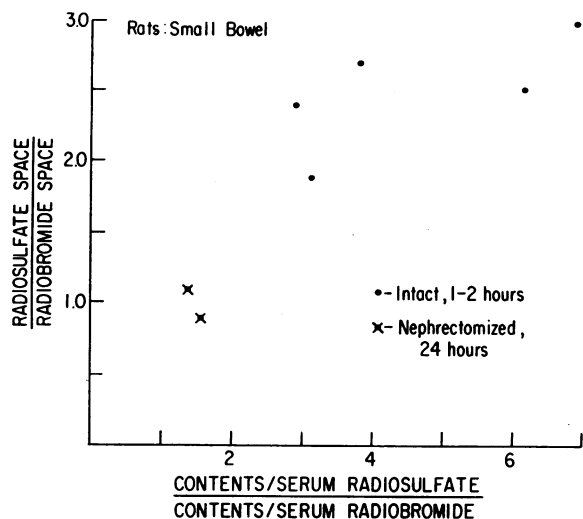


FIGURE 2 The ratio of radiosulfate space to radiobromide space in the wall of the rat small intestine as a function of this ratio in the intestinal contents. A positive correlation is seen. The ratios approach unity together, suggesting that the true ratio in the tissue is close to one.

ble I). Thus it would appear that inorganic sulfate, as such, is present intracellularly in the liver, or is extensively bound in the extracellular space. Alternatively, it is possible that our measurement may have included some labeled organic sulfate compounds (see Discussion). In an attempt to distinguish between these possibilities, the experiments were repeated in rats loaded with carrier sulfate (group IIB). If the amount of sulfate incorporated into organic form (and measured by our method) did not increase, it would become a smaller fraction of the measured tissue radioactivity; thus the sulfate space should decrease relative to bromide space. The results (Table I) reveal a significant reduction of both E^{35SO_4} and E^{82Br} , but not of the ratio E^{35SO_4}/E^{82Br} . Thus elevation of plasma inorganic sulfate does not

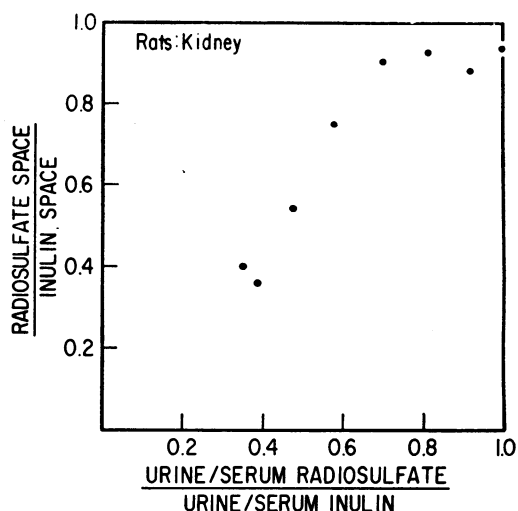


FIGURE 3 The ratio of radiosulfate space to inulin space in rat kidney, as a function of the analogous ratio in the urine, during increasing mannitol-sulfate diuresis. The abscissa represents the fraction of the filtered sulfate excreted, which approaches unity. The ratio of tissue spaces remains less than one, despite the fact that the contribution of tubular fluid to both spaces is equalized. Thus the ratio in kidney parenchyma, exclusive of tubular fluid, cannot be significantly greater than unity.

alter the distribution of $^{35}SO_4$ relative to ^{82}Br in the liver, but causes a slight reduction in the amount of extracellular fluid, probably attributable to the diuretic effect of Na_2SO_4 .

There is a significant difference between the two dogs in E^{35SO_4} and E^{82Br} , but not in E^{35SO_4}/E^{82Br} . The means have therefore been calculated for each dog, and in both, E^{35SO_4} significantly exceeds E^{82Br} by a proportion similar to that observed in the rats.

The location and chemical nature of the sulfate beyond the bromide space in liver cannot be finally ascertained from these observations, but the absence of a

TABLE II
Radiosulfate Space (E^{35SO_4}) and Inulin Space (E_{In}) of the Kidneys during Mannitol-Sulfate Diuresis

Rat No.	E^{35SO_4} *	$\frac{\text{Urine } ^{35}SO_4}{\text{Serum } ^{35}SO_4}$	E_{In} *	$\frac{\text{Urine inulin}}{\text{Serum inulin}}$	Ratio of E^{35SO_4}/E_{In}	Ratio of $\frac{U/S \text{ } ^{35}SO_4}{U/S \text{ In}}$
	% wet wt		% wet wt			
II C1	78	2.8	198	8.0	0.40	0.35
II C2	57	4.2	161	10.6	0.36	0.39
II C3	72	5.6	128	11.7	0.56	0.48
II C4	77	5.4	102	9.3	0.75	0.58
II C5	83	3.6	98	5.1	0.85	0.70
II C6	87	2.7	95	3.3	0.93	0.81
II C7	109	3.5	117	3.7	0.88	0.94
II C8	74	6.0	84	6.0	0.94	1.00

U, urine; S, serum; In, inulin.

* Mean of both kidneys.

TABLE III
Distribution of Inorganic Radiosulfate and Radiobromide in Whole Bodies of Rats

		A ₁	A ₂	A ₃	B ₁	A ₄	A ₅	B ₂	C ₁	C ₂	D ₁
Time after injection, hr		0.9	1.1	1.2	1.2	1.4	1.5	2.2	24.0	24.0	2.0
Procedure									Nephrex	Nephrex	Nephrex, SO ₄ load
Distribution of injected isotopes, % dose											
Gut contents	³⁵ SO ₄	3.1	3.1	2.2	3.8	5.6	14.8	4.9	3.4	4.9	3.7
	⁸² Br	6.5	10.1	8.7	6.8	7.5	3.8	7.4	11.1	16.3	4.9
Bladder urine	³⁵ SO ₄	8.1	11.5	3.3	2.4	22.2	30.7	14.6	—	—	—
	⁸² Br	0.3	0.8	0.1	0.8	0.8	0.9	3.0	—	—	—
External loss	³⁵ SO ₄	—	—	—	—	—	3.3	—	3.0	1.4	—
	⁸² Br	—	—	—	—	—	3.3	—	9.9	4.7	—
Total Carcass	³⁵ SO ₄	71.0*	66.6	76.8	70.7	47.3	40.6	48.6	39.1	45.4	90.4
	⁸² Br	93.3	89.1	91.1	92.5	91.7	92.0	89.6	79.0	79.0	95.1
Metabolized	³⁵ SO ₄	17.8	18.8	17.7	23.1	24.9	10.6	31.9	54.5	48.3	5.9
Apparent volume of distribution, V, corrected for urinary and external losses, ml/100 g body wt											
	V ³⁵ SO ₄	36.3	33.5	38.8	37.1	38.5	42.8	49.2	61.1	50.9	27.7
	V ⁸² Br	29.0	30.0	33.6	32.6	29.6	27.3	33.8	32.5	34.5	28.4
	V ³⁵ SO ₄ /V ⁸² Br	1.25	1.12	1.15	1.14	1.30	1.57	1.46	1.88	1.48	0.96
Measured space, E, ml/100 g body wt											
Gut contents	E ³⁵ SO ₄	1.2	1.2	0.9	1.4	2.8	8.6	2.8	2.2	2.5	1.0
	E ⁸² Br	1.9	3.1	2.9	2.2	2.4	0.7	2.6	4.0	5.9	1.4
Viscera†	E ³⁵ SO ₄	—	—	—	7.2	—	—	6.0	4.1	3.7	—
	E ⁸² Br	—	—	—	5.9	—	—	4.3	3.9	4.0	—
Eviscerated carcass	E ³⁵ SO ₄	—	—	—	19.7	—	—	22.0	19.5	19.7	—
	E ⁸² Br	—	—	—	24.5	—	—	27.0	24.6	24.6	—
	E ³⁵ SO ₄ /E ⁸² Br	—	—	—	0.80	—	—	0.81	0.79	0.80	—
Total carcass	E ³⁵ SO ₄	28.0*	25.2	30.8	26.9	23.4	26.3	28.0	24.6	23.4	25.0
	E ⁸² Br	27.1	27.0	30.6	30.4	27.3	26.7	31.3	28.5	28.6	27.0
	E ³⁵ SO ₄ /E ⁸² Br	1.03	0.93	1.01	0.88	0.86	0.98	0.89	0.86	0.82	0.92
Total carcass, including gut contents	E ³⁵ SO ₄	29.2*	26.4	31.7	28.3	26.2	34.9	30.8	26.8	25.9	26.0
	E ⁸² Br	29.0	30.1	33.5	32.6	29.7	27.4	33.9	32.5	34.5	28.4
	E ³⁵ SO ₄ /E ⁸² Br	1.01	0.88	0.95	0.87	0.88	1.27	0.91	0.82	0.75	0.92

* Corrected for prolonged extraction (see Methods).

† Liver, spleen, gastrointestinal tract, kidneys and bladder.

change in this quantity with time and the fact that it increases in proportion to extracellular sulfate both militate against a metabolically dependent uptake or conversion.

Small bowel. The observed values of E³⁵SO₄ and E⁸²Br in the gastrointestinal samples are influenced by contamination with luminal contents. Thus the low concentration of ³⁵SO₄ relative to ⁸²Br in the gastric contents results in a low value of E³⁵SO₄/E⁸²Br in the stomach wall, and no deductions about the intracellular ⁸²Br concentration can be made. Conversely, the discrimination of the small bowel contents in favor of ³⁵SO₄ results in a radiosulfate space that exceeds the radiobromide space in the small intestinal wall in the rat. As shown in Fig. 2, there is a relationship between E³⁵SO₄/E⁸²Br in the small bowel and the luminal discrimination between the

two ions which suggests that the true value for E³⁵SO₄/E⁸²Br in the absence of contamination would be close to unity. In dog 2 we found E³⁵SO₄ of the small bowel to be less than E⁸²Br. The reason for this apparent species difference may be that the dogs were fasting whereas the rats were not.

Kidney. The urinary clearance of radiosulfate exceeds that of radiobromide, and therefore it was not unexpected to find that renal E³⁵SO₄ exceeded E⁸²Br; we attributed this to the contained tubular fluid. A roughly linear relationship between E³⁵SO₄/E⁸²Br (y) and the ratio of the renal clearance of ³⁵SO₄ to ⁸²Br (x) was found described by $y = 1.2 + 0.01x$. In order to elucidate the distribution of inorganic ³⁵SO₄ in the kidney, we compared the renal radiosulfate space to the inulin space, E_{in}, under conditions of mannitol and sulfate diu-

resis in rats (group IIC, Table II). Mannitol loading diminishes the tubular fluid/serum ratio of inulin and sulfate, and therefore minimizes the contribution of trapped tubular fluid to the measured spaces. Sulfate loading, by saturating the reabsorptive mechanism for inorganic sulfate, causes the sulfate clearance to approach that of inulin and therefore tends to equalize the contribution of trapped tubular fluid to the two spaces. Fig. 3 shows that even when the sulfate clearance approaches the inulin clearance, renal $E^{35\text{SO}_4}$ is less than E_{in} . These data suggest that, under these conditions, the concentration of inorganic $^{35}\text{SO}_4$ in the renal cell is negligible.

Knee. $E^{35\text{SO}_4}$ exceeds $E^{82\text{Br}}$ in the knee, and the absolute value of the spaces, as well as the ratio $E^{35\text{SO}_4}/E^{82\text{Br}}$ was not significantly altered by sulfate loading (Table I). This result, like that in liver, does not exclude the possibility of intracellular uptake or metabolic alteration, but is more suggestive of passive binding of sulfate.

Radiosulfate and radiobromide spaces of the rat carcass

In Table III are summarized the results of analyses of 10 rats. Nephrectomy was performed before isotope injection in three, of whom one was loaded with carrier sulfate. The first line shows the amounts of each isotope found in the contents of the gastrointestinal tract. In the seven normal animals, an average of 5.4% of the injected radiosulfate and 7.3% of the radiobromide was recovered. The difference is not statistically significant, but in six of the seven rats, the ^{82}Br content exceeded the $^{35}\text{SO}_4$ content, as it also did in the three nephrectomized rats. In view of the analyses of the contents of different portions of the gut shown in Table I, we infer that the contribution of gastric juice with its low sulfate content outweighs the contribution of small and large bowel contents. In the nine rats not given carrier sulfate, the fraction of injected ^{82}Br found in the gut increases significantly with time ($P = 0.01$), but that of $^{35}\text{SO}_4$ does not.

The bladder contained very little radiobromide but considerable quantities of radiosulfate, as anticipated from the greater clearance of sulfate. Some increase with time is apparent for both isotopes. The line designated "external loss" includes urine and feces passed in the cage, vomitus, saliva, and some blood. These were negligible except in the two nephrectomized rats and one other.

The lower half of Table III shows the apparent volumes of distribution, corrected for urinary and external losses, of the two isotopes, and the breakdown of those volumes in terms of the measured spaces, defined as the amount present divided by the intestinal fluid concentration, in the gastrointestinal contents, viscera, and

carcasses. The total carcass space of each isotope, excluding gastrointestinal contents, shows no increase with time between 0.9 and 24 hr, nor does the ratio of the spaces. Excluding the sulfate-loaded animal, the mean values are: $E^{35\text{SO}_4} = 26.3\% \pm 0.8\%$ body weight, $E^{82\text{Br}} = 28.6\% \pm 0.6\%$, and $E^{35\text{SO}_4}/E^{82\text{Br}} = 0.92 \pm 0.02$ (SEM, $n = 9$). Including the gastrointestinal contents, the observed values for the carcass spaces are: $E^{35\text{SO}_4} = 28.9\% \pm 1.0\%$ body weight, $E^{82\text{Br}} = 31.4\% \pm 0.8\%$, and $E^{35\text{SO}_4}/E^{82\text{Br}} = 0.93 \pm 0.05$ (SEM, $n = 9$).

These results, when compared with the data in Fig. 1, emphasize the difference between the fate of these two isotopes. Because of the method used to correct for carcass losses during processing, the bromide carcass space, including gastrointestinal contents, is equal to the apparent volume of distribution. The bromide volume of distribution shows no increase with time; in contrast, the sulfate volume of distribution significantly overestimates the true inorganic sulfate space, owing to metabolic conversion of sulfate to organic forms. The portion of the dose of the isotope not recovered in inorganic form, as shown in Table III, is already about 18% at 1 hr, and increases subsequently. This result supports the suggestion (8) that a pool of organic sulfate exists in the rat into which injected radiosulfate enters rapidly.

In the one sulfate-loaded rat, the fraction of injected isotope metabolized was considerably lower as expected, but the ratio of spaces was the same (0.92). In this animal the ratio of the apparent volume of distribution of sulfate to that of bromide was 0.96, compared with expected ratio of 1.32 at this interval in non-sulfate-loaded animals (Fig. 1). This difference is fully explained by the smaller fraction metabolized. The unchanged ratio of carcass spaces supports the inference that all of the sulfate measured in these carcasses has achieved its distribution by passive processes, not subject to saturation at higher sulfate concentration.

In the four rats in which the viscera and the eviscerated carcasses were analyzed separately, the ratio of radiosulfate space to radiobromide space in the eviscerated carcasses was much more constant. In a fifth rat (not shown in the Table), in whom only the eviscerated carcass was analyzed two hr after the injection of isotopes, a ratio of 0.79 was obtained. The mean of the five values is 0.789 ± 0.004 (SEM). These values are more constant because the variable contributions of gut and kidney are absent. The combined viscera themselves, comprising $11\% \pm 1\%$ (SEM, $n = 4$) of body weight, have high proportions by weight of both sulfate and bromide spaces, especially the former.

DISCUSSION

These experiments show that inorganic radiosulfate rapidly achieves an equilibrium volume of distribution in

several individual tissues of rats and dogs and in the rat carcass. The only time-dependence we observed was in the amount of bromide within the gut contents. A constant radiosulfate space in rat and dog skeletal muscle was also achieved between 1/2 and 24 hr, reported in detail elsewhere (9). Thus the distribution of radiosulfate, as measured in these experiments, probably corresponds closely to the distribution of inorganic sulfate in the intact animal. If unmeasured pools of inorganic sulfate exist, they must be very small in magnitude, slow in exchange rate, or both.

On the other hand, the possibility that inorganic radiosulfate has been overestimated cannot be excluded. There are two ways in which this might have occurred. Organic sulfates could be decomposed during the analytical procedures or could be measured along with inorganic sulfate. The analytical procedures were designed specifically to reduce these dangers. Thus, those tissues and carcasses which were dried by lyophilization were frozen within seconds and were not exposed to temperatures approaching zero until dry; furthermore, the protein precipitants were only weakly acid ($\text{pH} > 4$). Nevertheless labile organic sulfates, containing ^{35}S , may have broken down. Furthermore, some mucopolysaccharide sulfates may be precipitated by benzidine (10), and these too may have contained ^{35}S . The former possibility may explain the strikingly high results in liver, and the latter the less prominently high values in cartilage, exemplified by the knee. It may be noted in passing that barium as a precipitant for inorganic sulfate also lacks specificity: mucopolysaccharides may be coprecipitated under certain conditions (11). Evidently, only chromatographic or electrophoretic separation can provide a clear isolation of inorganic sulfate (12).

Analytical difficulties clearly do not account for the high ratio of sulfate to bromide space in kidney and gut of the rats. In these tissues, the transcellular fluids represent precursor solutions, similar in ionic composition to interstitial fluid, from which salt and water have been reabsorbed to a variable extent. As a result of the low permeability of these epithelial membranes to sulfate compared with the halides, radiosulfate becomes progressively concentrated relative to radiobromide. A relationship between the extent of this concentrating process in both tissues and the observed ratio of radiosulfate to radiobromide spaces was demonstrated. The opposite type of discrimination is seen in the stomach (Table I). Here sulfate concentration is lower than in intestinal fluid, owing to the fact that it does not participate in the anion-transporting mechanism which leads to the elaboration of the acid gastric secretion, as shown in Table I.

Bromide also achieved a constant volume of distribution in all of the tissues examined, including muscle (9). This indicator was chosen in preference to stable

chloride because of its ease of analysis. Conventional tissue chloride methods have been made suspect by the observations of Cotlove (13, 14) that pronounced overestimation of chloride by argentometric methods may occur as a result of sulfhydryl groups derived from tissue proteins. Whether radiobromide distribution accurately reflects chloride distribution is not entirely clear. It evidently does so in muscle (6), erythrocytes (15), and several other tissues (16-18), but not in brain (16-18). Gastrointestinal juices equilibrate almost completely in one day (15). Bromide space in whole animals closely approximates chloride space (19-22). Thus in most tissues we have examined it can be assumed that the equilibrium distribution of radiobromide corresponds closely to that of chloride.

In skin and in tail, radiosulfate space was about 10% less than radiobromide space. These observations, though few in number, cast considerable doubt on the view (23-25) that connective tissue contains exceptionally large quantities of bound chloride. Similar observations were made by Kavalier (26) in tendon and by Nichols, Nichols, Weil, and Wallace (24) in skin, using thiosulfate. Langgård (27) recently reported that 15% of skin chloride exchanges at a slow rate.

The absence of selective inorganic sulfate concentration in skin and bone stands in sharp contrast to the distribution of labeled sulfur after $^{35}\text{SO}_4$ administration (28). Mellick and Cavanagh (29) report a sulfate space of skin of 71% of wet weight, using a method which measures total ^{35}S . These tissues evidently accumulate sulfate chiefly in organic form, and contain little or no bound inorganic sulfate. In the knee, however, sulfate space clearly exceeds bromide space, even after loading with carrier sulfate. The difference is presumably attributable to cartilage, present in large amounts in these samples. Katsura and Davidson (30) have analyzed the sulfate-containing components of cartilage by chromatography and have found that inorganic sulfate tends to migrate with polysaccharide components, and can be removed only with difficulty. They suggest that inorganic sulfate as such is taken up by cartilage at sites already occupied by ester sulfate. This hypothesis is consistent with our data if additional unoccupied sites are available for binding when the plasma sulfate level is substantially increased.

Owing to contained transcellular fluids, the gut and kidney ratios of sulfate space to bromide space can provide little information as to the quantity of nonextracellular chloride or sulfate. However, in both tissues the results are consistent with the possibility that sulfate space is equal to or less than chloride space in the tissue proper, excluding the transcellular fluids.

It is difficult to reconcile our findings with the work of Deyrup and Ussing (32) and Berglund and Deyrup (33), who have shown that kidney slices accumulate large

TABLE IV
Distribution of Total Body Halide in Rats

	(1) Organ wt, fraction of body wt	(2) Bromide space, fraction of organ wt	(3) Maximal ex- tracellular space, fraction of organ wt	(4) Fraction of total body halide*
Intracellular or bound				
Muscle	0.45 (36)	0.117 (9)	0.095 (9)	0.033
Skin	0.12 (25)	0.43‡	0.38‡	0.022
Erythrocytes	0.032 (36)			0.061 (36)
Connective tissue				0.015 (36)
Transcellular				
Kidney	0.008‡	0.050‡	0.30 (31)	0.006
Stomach wall	0.007‡	0.54	0.28‡	0.006
Small bowel wall	0.042‡	0.20‡	0.12§	0.011
Gut contents				0.073‡
Total nonextracellular				0.227
Extracellular				0.773
Total				1.000

* Calculated as $[(2) - (3)] \times (1) \div 0.307$ (total bromide space, including gut contents, as a fraction of body wt).

‡ Measured in this study.

§ Calculated from radiosulfate space as explained in text.

amounts of ³⁵S when placed in solutions containing radio-sulfate. The characteristics of sulfate accumulation by slices are similar to those of the sulfate-transporting mechanism in the intact kidney. Thus it is conceivable

that transport into the cells persists in these incubated tissues but that efflux from the cells is impaired.

Liver consistently exhibited the highest ratio of sulfate to bromide space in both species. The possibility that

TABLE V
Distribution of Inorganic Sulfate in Rats

	(1) Organ wt, fraction of body wt	(2) Sulfate space, fraction of organ wt	(3) Maximal extra- cellular space, fraction of organ wt	(4) Fraction of total body sulfate*
Intracellular or bound				
Liver	0.038‡	0.36‡	0.23‡	0.018
Cartilage	0.003§	0.25‡	0.22‡	0.003
Erythrocytes	0.032 (36)	0.35	0.00	0.040
Transcellular				
Kidney	0.008‡	0.70‡	0.30 (31)	0.011
Small bowel wall	0.042‡	0.42‡	0.12	0.045
Gut contents				0.054‡
Total nonextracellular				0.171
Extracellular				0.829
Total				1.000

* Calculated as $[(2) - (3)] \times (1) \div 0.28$ (total inorganic sulfate space, including gut contents, as a fraction of body wt).

‡ Measured in this study.

§ Guessed.

|| Calculated as in Table IV.

this finding is attributable to analytical errors has been discussed, but the identical results obtained after loading with carrier sulfate are somewhat against this interpretation. Since liver is one site of formation of "active sulfate" (34), it is conceivable that a mechanism exists for accumulating inorganic sulfate intracellularly. Furthermore, isolated mitochondria have been shown to possess the capacity to accumulate sulfate (35), presumably in inorganic form.

The distribution of bromide and sulfate in the rat may be summarized as in Tables IV and V, respectively. The distribution of bromide closely resembles the distribution of chloride (see above); hence these results extend and modify the earlier conclusions of Cheek, West, and Golden (36). The "maximal" extracellular space of muscle, skin, and stomach wall, shown in Table IV, has been estimated from the radiosulfate space. If appreciable inorganic radiosulfate penetrates the cells of these organs, the intracellular bromide would be larger. In Table V, the maximal extracellular space of liver and cartilage has been estimated from the bromide space. The quantity of cartilage in the body is conjectural, but the amount of nonextracellular sulfate therein is small. In both tables, the extracellular space of the kidney (excluding tubular fluid) has been taken as 30%, in accordance with the findings of Gärtner (31). The maximal extracellular space of the small intestine was calculated from the data in Table I on the assumption that both bromide and sulfate spaces of the gut wall comprise two moieties: a true extracellular space, equal in volume for both $^{35}\text{SO}_4$ and ^{82}Br , and trapped luminal fluid, in which the ratio of sulfate space to bromide space is the same as in the luminal contents, namely, 3.7. This leads to an estimate of 12% of wet weight for the true extracellular space of this tissue.

The results of these calculations, which clearly must be viewed as approximations, show that neither ion is confined to the extracellular space. At least 23% of body chloride and at least 17% of body sulfate is nonextracellular. Our figure for nonextracellular chloride is considerably higher than that of Cheek and associates (36), namely, 12.7%. The difference is attributable to the fact that these authors give intracellular chloride in muscle as zero, and chloride in gut contents as only 2.4% of the total. Sweet, Nadell, and Edelman (15) found 16% of body chloride in the gut in rabbits, but only 1.9% in human subjects examined postmortem. Evidently this value may vary considerably, both between and within species.

A maximum estimate of the "true" extracellular space in the rat can be calculated as the product of the fraction of bromide or sulfate that is extracellular (from Tables IV and V) and the total carcass bromide or sulfate space including gastrointestinal contents (from

Table III). The results are 24.3% and 24.0% of body weight, respectively. The close correspondence between these two values may be partly fortuitous, but the true value must be close to these estimates.

Insufficient data are available to make comparable calculations for other animals or for man. The chief uncertainties are the intracellular chloride in muscle and transcellular sulfate in the gut. Both quantities can readily be determined in vivo.

The implications of this work with respect to the measurement of extracellular space in human subjects are of interest. The large difference between sulfate space and bromide or chloride space in man (37) can no longer be attributed to "nonfunctional" portions of the extracellular space, in the sense that longer equilibration would lead to further penetration of the labeled ions. The chloride beyond the limits of the sulfate space appears to be intracellular (or transcellular), in amounts which vary widely from one tissue to another and which are often too large to account for by passive distribution of chloride across the membranes of cells other than the erythrocytes. It does not follow, however, that the non-extracellular chloride is unresponsive to change in plasma chloride; in fact, the available evidence suggests the opposite conclusion (38). The appropriate measure of extracellular space in the whole organism can thus be settled only by establishing physiological correlations with any proposed technique. The "true" extracellular space is closer to sulfate space than bromide space, though smaller than either, but the "functional" volume may be different. In the presence of circulatory impairment or edema, the virtual constancy of bromide distribution after equilibration has been achieved is a considerable advantage.

Various techniques for analyzing plasma and urinary concentration curves for radiosulfate have been suggested and interpreted in terms of a limited number of pools or compartments (39). Our observations indicate that the complexity of distribution of sulfate precludes the use of any simple mathematical model.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health, U. S. Public Health Service (AM-02306). Dr. Walser is supported by U. S. Public Health Service Research Career Award Program (GM-K3-2583).

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