THE HIGH SPECIFICITY OF THE MANGANESE PATHWAY THROUGH THE BODY [1, 2]

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The accepted ideas about the physiological role of manganese have been derived predominantly from *in vitro* experiments. However, these are characterized by lack of specificity: Only a limited discrimination between manganese and some other metals is shown, for instance, in metal transport systems (1, 2), in enzyme activation (2–4) and in the preservation of mitochondria (5). The conclusions from these experiments were of interest because they threw new doubt on the functional specificity and even on the essentiality of manganese in the living organism. Thus it became necessary to ascertain whether manganese may be replaced in the intact animal as well. To investigate this, we have used a variation of the classical technique of flooding the organism with metals which we were led to believe would substitute for manganese. Surprisingly, these kindred metals were ineffective in eluting radiomanganese from the body: Only manganese compounds proved effective in that regard.

The results suggest that there exists a segment in the pathway of manganese through the body, the properties of which permit the passage of that metal only. This paper deals with the implications of this finding both in reference to manganese as well as to the elements which have been thought not to possess such an *in vivo* specificity.

MATERIALS AND METHODS

Experiments with intact animals

Animals. These were seven week old Swiss albino male mice, weighing 16 to 18 grams. They were housed in cages, the bedding of which consisted of shredded corn cobs. When dietary restrictions were imposed, metal screening was used to prevent the animals from eating the bedding. Regardless of the type of bedding, the furs remained radiologically clean, as shown by the failure of Versene® and detergent baths to lower the total body radioactivity.

Diets. Since the manganese turnover depends upon dietary intake, an effort was made to keep the manganese intake constant. In the bulk of the experiments, the same lot of Purina Fox chow was used ($MnSO_4$ reported by manufacturer as 0.02 per cent). As a "manganese free" diet, a vitamin B_{12} deficient rat diet was used.[3] To this a salt-vitamin fortification mixture[4] was added without supplementary manganese, unless otherwise stated.

Stable metals and their salts. Rhenium dioxide was prepared by dissolving the metal in nitric acid and heating to dryness with anhydrous hydrazine HCl. The procedure was repeated twice with the precipitate. This was finally washed with water, ground into a fine suspension in normal saline and injected into the animals intraperitoneally. All the metals, oxides and salts[5] used in these experiments to challenge the isotope were injected with normal saline as the vehicle. In some experiments (see Table I) the sulfates or chlorides of the metals were dissolved with added equimolar amounts of sodium citrate (hereafter referred to simply as "citrate"). In those latter experiments the controls received equimoler sodium citrate instead of the routinely used saline injections. The doses of the challenging materials ranged from 1×10^{-6} to 1×10^{-5} mole of metal. These were given either as single or as daily injections for one week.

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[2] A preliminary report of this paper was presented befor the Federation of American Societies for Experimental Biology in Chicago, March, 1957.

[3] Nutritional Biochemicals Corporation. The complete diet contained 2.5γ of manganese per 100 gram diet (or 1γ of manganese per gram ash).

[4] Nutritional Biochemicals Corporation, "Vitamin Diet Fortification Mixture."

[5] Spectrographic tests of the $MnSO_4$ powder showed it to be free from contaminating metals, while both the $FeSO_4$ and the $CrCl_2$ powders contained 0.01 per cent manganese. The utilized chemical agents and their sources of purchase are as follows: cobaltous sulfate, ferrous chloride, manganese dioxide, nickelous sulfate and sodium citrate from Baker and Adams Co.; copper sulfate, magnesium sulfate, potassium permanganate and zinc sulfate from Baker's; ferric chloride from Merck & Co.; manganous chloride from Mallinkrodt; manganese metal from A. D. Mackay; vanadyl sulfate from Fisher; and vanadium metal from Amend Drug. The rhenium compounds were obtained from the University of Tennessee except for the rhenium pentachloride (K & K Labs.).

Isotopes. Mn^{52} ($T/2 = 5.7$ days) was prepared by the Cr^{52} (d, 2n) Mn^{52} reaction in a 60 inch cyclotron by one of us (J. J. G.). Separation of the tracer Mn^{52} from the chromium necessitated a precipitation with manganese carrier. When the latter was found to have a marked influence on the phenomena under study, this preparation was abandoned in favor of Mn^{54}. The latter isotope was prepared carrier free by the Nuclear Science and Engineering Corporation. It was received as $Mn^{54}Cl_2$ in hydrochloric acid (200 μc. per ml.). Neutral dilutions (0.8 and 1.6 μc.) were given intraperitoneally to the animals in 0.1 or 0.2 ml. of saline. While with the Mn^{52} variations even from 0.1 to 0.2 μc. brought upon concordant variations of the turnover rate, the Mn^{54} preparation was free from such complicating effects. Accidental injections into a hollow viscus were soon followed by almost complete loss of the animal's radioactivity.

Measurements of the radioactivity. Each tagged animal was placed in a 50 ml. plastic centrifuge tube with its mouth opposite to the outlet of an oxygen line. After plugging with cotton, the tube was inserted into the horizontal well of a Texaco® counter. The same procedure (minus the ventilation) was used for pooled organs, to permit the direct comparison between animals and their organs. As a rule counting was accurate to within 1 per cent in both cases, and its geometry was constant between 3 and 30 ml.

Calculations of turnover rates. The animal's total body radioactivity was counted within one hour after isotope injection. This was repeated daily, in some cases for as long as 20 to 30 days. The subsequent counts were expressed as percentages of the first, after being corrected for background and, if needed, for isotopic decay. The data were then plotted on semilogarithmic paper as a function of time and the curves were drawn (Figure 1). When necessary, these were analyzed graphically for their constituent components (Figure 2) by subtracting the straight end from the remainder of each curve (6). In the experiments in which challenging injections of carrier metals were given, the following controls were used: *1)* saline- or sodium citrate-challenged animals; *2)* each animal's own prechallenge turnover rate; *3)* animals that had received $MnCl_2$ in amounts often less than equimolar to that of the challenging metal. At the end of some experiments, in which the challenge did not effect the turnover rate, the isotope's continued availability for exchange was demonstrated by injecting $MnCl_2$ solutions.

Summary of typical protocol. Thirteen mice were injected intraperitoneally with 1.0 μc. of $Mn^{54}Cl_2$ in 0.2 ml. saline, and their total body radioactivity determined. They were then divided into four groups. The retained radioactivity was measured at daily intervals. The corrected counts were plotted semilogarithmically against time as per cents of the first count. On the third day, Group I received 1×10^{-6} mole of $CrCl_2$; Group II, 0.2 ml. of saline; Group III, 2×10^{-7} $MnCl_2$; Group IV, 1×10^{-5} $FeCl_2$. The day following the injection the phenomenon illustrated in Figure 4 was seen. It was followed for five days and then the 2×10^{-7} mole of $MnCl_2$ were given to the animals that had received $CrCl_2$ and $FeCl_2$. This was done in order to test whether their tracer had remained exchangeable. The animals were then sacrificed, and the peritoneal cavities were found to be free of metallic deposits.

OBSERVATIONS

Turnover of body manganese. The results shown in Figure 1 were obtained with a group of nine Mn^{54} tagged animals. These animals were maintained on Purina Fox chow. The individual data fell into exceedingly smooth curves so that. subsequently, breaks in the curves which were experimentally induced could be used as additional controls. When these curves were graphically analyzed, at least two components were found ($T/2 = 48$ to 68 hours and $T/2 = 230$ to 300 hours).

Two groups of three mice each were given 1 per cent ammonium chloride and 1 per cent sodium bicarbonate instead of drinking water. After no effect on the turnover was seen, their bottles were switched. This again failed to produce any change in the turnover rates.

Effect of diet on manganese turnover. The feeding of the "manganese-free" diet to a group of three mice a few days prior to and during the experiment did not effect the rate of the first component ($T/2 = 54$ hours), while that of the second increased to $T/2 = 860$ hours. The diet was enriched with carrier manganese sulfate (0.01 per cent of Mn^{++} w/w), and the procedure was repeated in three other mice. This showed turnover

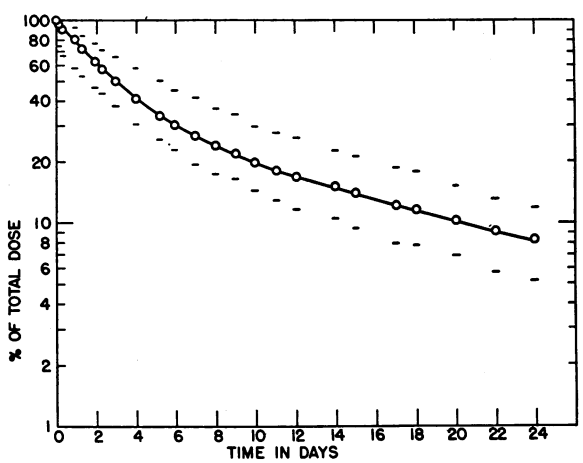

FIG. 1. TOTAL BODY TURNOVER OF Mn^{54} IN MICE (SEE TEXT)

The dashed lines define the envelope of all the data.

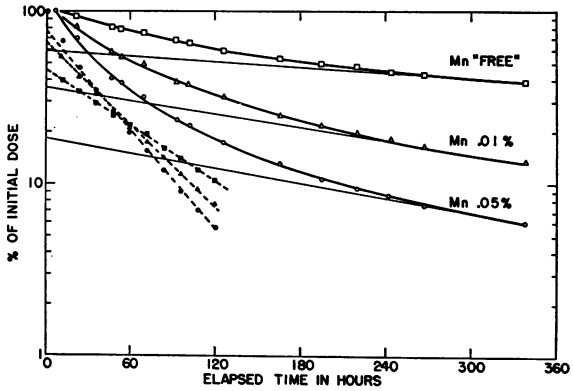

FIG. 2. TOTAL BODY TURNOVER OF Mn^{54} AS INFLUENCED BY DIETARY MANGANESE

rates of T/2 = 38 and T/2 = 238 hours for each of the two components. Increase of the manganese supplement to 0.05 per cent caused further decreases of these numbers to T/2 = 31 and T/2 = 208 hours in another group of three mice. Starvation of three animals markedly decelerated the turnover. Obstruction of the intestine at the anus in another three mice stopped the turnover entirely.

These experiments show that the rate of radiomanganese loss from the body is sensitive to the level of dietary manganese and to the state of gastrointestinal function. They are compatible with intestinal excretion of this metal (*7*). Concordantly, the acceleration of the observed turnover by added dietary manganese might have had the following implications: *1*) that more carrier manganese is absorbed from the gut than hitherto suspected and elutes the radioisotope from the tissues; or *2*) that on elimination from the gut, the stable metal carries with it a significant fraction of the bile-bound tracer (*6*, *7*) which normally might have been reabsorbed (*8*). Regardless of which explanation might be correct, this uncertainty would throw doubt on experiments with any stable metal, if these were fed. Thus the oral route was abandoned in favor of the intraperitoneal.

Accelerated elimination of radiomanganese following challenge with manganese carrier. Both Mn^{54} and Mn^{52} injected animals were used in these experiments.

As can be seen in Table I, various forms of the stable metal were given at various times. The valence state of the carrier ranged from 0 in the metal powder to 7 in the permanganate. Injections of all of these compounds were followed by acceleration of the rate of turnover, which resulted in losses ranging from 15 per cent to 80 per cent within twenty-four hours. Figure 3 illustrates two characteristic experiments at two different postinjection times with two different isotopes. By varying the time at which the

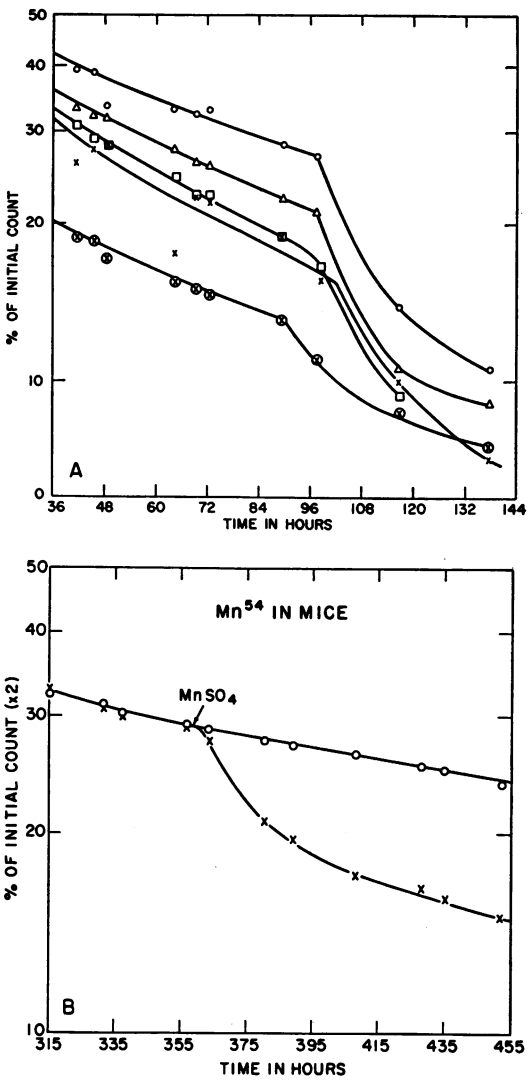

FIG. 3. EFFECT OF ADDING CARRIER MANGANESE ON RADIOACTIVE MANGANESE TURNOVER

A = Total body turnover of Mn^{52} in mice receiving 8×10^{-6} mole of carrier manganese as $MnSO_4$ at the 86th hour of the experiment.

B = Averages of total body turnovers of Mn^{54} in three mice compared with those of three mice receiving 4×10^{-6} mole of carrier manganese as $MnSO_4$ at the arrow.

challenge was made, it was found that an appreciable fraction of the tracer had remained exchangeable, even three weeks after tagging the animals. The exchange has not been quantitated as yet. The impression was gained, however, that elution of the tracer manganese was more obvious when the challenge was made early or with high doses of carrier. An easily observed acceleration of the turnover was evident following injections of 9×10^{-8} mole of $MnSO_4$ as late as the sixth day. The extreme valence states (Mn^0 and Mn^{7+}) seemed less effective than Mn^{2+}.

These experiments proved that the radioisotope remained largely and continuously available for elution by carrier. The elution was obvious after challenges with $MnSO_4$ amounting to 30 per cent of the estimated manganese content of the body.

Failure of some other stable metals to affect Mn^{54} turnover. Manganese is a member of both the first transition group and of group VIIB of the elements. Many of the members of the transition group (in periods four and five) have been known to substitute for manganese *in vitro* (1–5, 9). Manganese and rhenium are the only stable members of group VIIB. Rhenium is Mendeleef's celebrated dvi-manganese (10). His ekamanganese (technetium), which would have been very crucial in these studies, has only radioactive isotopes.

These metals were used as eluting agents in experiments identical with those described in the case of carrier manganese. Magnesium, the element most widely used *in vitro* instead of manganese, was also included. In another experiment, a member of group VIIA was tested because of its possible interchange with manganese in the thyroid (11).

The upper limit of the concentration in the challenging injections was set by the toxicity of the individual compounds. Safe doses were chosen after preliminary experimentation with groups of mice. Care had to be taken to avoid interference with the animals' eating and bowel habits which were known to affect the end point.

The lower limit was set at 30 per cent of the mouse's total body content of each of the stable metals used as eluting agents. This value was chosen because of the effect of carrier manganese described above. Furthermore, 30 per cent of anything appeared to be a large amount. Table I shows both the metals' estimated body content and the challenging doses given. High values were intentionally chosen in making these estimates. A mouse weighing 20 grams was chosen as a prototype in spite of the fact that the experimental animals weighed 16 to 18 grams. Even so, the desired value of 30 per cent was reached or exceeded by the individual injections. It was markedly surpassed in the experiments in which the injections were given daily for one week.

Every effort was made to compare similar valence states with each other. Some animals which had received toxic doses of ferrous and chromous chlorides had small peritoneal metallic desposits at autopsy. The mouse peritoneum had shown a high absorptive capacity in the experiments with manganese metal and dioxide powders. In spite of this the precaution was taken of including citrate in many of these injections, since this markedly facilitates absorption by forming chelates with these metals (12). The metals' release in the body was expected because citrate is rapidly metabolized.

These stable metals were incapable of affecting in any way the rate of elimination of radiomanganese from the body, in spite of all the specifications listed above. Figure 4 illustrates the failure of ferrous and chromous citrates to bring upon any change of the radiomanganese turnover in concentrations 50 times higher than an effective amount of manganous sulfate. That the isotope was available for exchange in these animals also was shown by the effect of the manganous salt at the end of that observation.

Experiments with organs

Following the observation that challenges with carrier manganese had caused elution of radiomanganese readily, reproducibly and regardless of the state of the carrier, the failure of any of the other stable metals to do so seemed incongruous. The following explanation for the apparent incongruity was considered and tested: It was thought that the nonmanganese metals might have caused elution of the tracer from one organ into another instead of elimination out of the body. This would not have been detected by our method, since it was designed to quantitate the radioactivity of the entire body and not to differentiate be-

TABLE I

Summary of metals administered to manganese^{54} injected mice

No. of mice	Metal or salt	Atomic number	Moles per 20 Gm. mouse*	Moles per challenge	$\Delta t\ \bar{p}$ isotope
7	$MgSO_4$	12	4.2×10^{-4}†	$5 \times 10^{-5} - 5 \times 10^{-4}$	146
3	$VOSO_4$	23	1.3×10^{-7}‡	1.0×10^{-5}	310–505§
9	Cr Cit	24	3.1×10^{-7}‡	$2 \times 10^{-6} - 1 \times 10^{-5}$	(−)6–505§
13	$CrCl_2$	24		$4 \times 10^{-6} - 1 \times 10^{-5}$	50–650
3	Mn^0	25	2.8×10^{-7}‡	$9 \times 10^{-5} - 4.5 \times 10^{-4}$	6–146
11	Mn Cit	25		$1.8 \times 10^{-6} - 9 \times 10^{-6}$	(−)6–674§
28	$MnSO_4$	25		$9 \times 10^{-8} - 1 \times 10^{-5}$	50–650
3	MnO_2	25		1.1×10^{-4}	(−)24
3	$KMnO_4$	25		2.5×10^{-6}	354
6	$FeCl_2$	26	2.1×10^{-5}‖	$9 \times 10^{-6} - 1 \times 10^{-5}$	50–650
11	Fe^{++} Cit	26		1.8×10^{-6}	(−)6–480§
3	Fe^{+++} Cit	26		1×10^{-6}	1
3	$FeCl_3$	26		3×10^{-6}	48
6	Co Cit	27	9×10^{-8}‡	$1.7 \times 10^{-6} - 8.5 \times 10^{-6}$	311–480§
3	$CoSO_4$	27		3.5×10^{-7}	266
3	Ni Cit	28	6.1×10^{-7}‡	$1.7 \times 10^{-6} - 8.5 \times 10^{-6}$	311–480§
7	Cu Cit	29	6.3×10^{-7}‖	$1 \times 10^{-7} - 3 \times 10^{-7}$	(−)6–364§
19	$CuSO_4$	29		$1 \times 10^{-7} - 6 \times 10^{-7}$	50–318§
3	Zn Cit	30	1.6×10^{-5}‡	$1.5 \times 10^{-6} - 7.6 \times 10^{-6}$	311–480§
3	$ZnSO_4$	30		3.5×10^{-7}	266
3	KIO_3	53	7.1×10^{-8}†	4.6×10^{-6}	354
7	Re^0	75		5.5×10^{-5}	6–50
7	ReO_2	75		4.6×10^{-6}	(−)24–270§
3	Re_2O_7	75		9.7×10^{-6}	1
3	$NaReO_4$	75		2.7×10^{-5}	354

* These are estimates of the mouse's total body content prior to challenge. All estimates pertain to the individual metal involved in the test and not to the salt listed.
† Estimated from Hawk, Oser and Summerson (14).
‡ Estimated from Tipton and co-workers (18).
§ Include daily injections for one week.
‖ Estimated from Underwood (9).

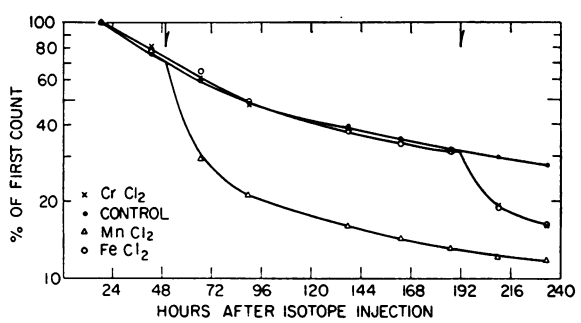

FIG. 4. TOTAL BODY TURNOVER OF Mn^{54} IN MICE AS INFLUENCED BY SOME CARRIER METALS

At first arrow 0.2 ml. of saline, 1×10^{-5} mole of either $FeCl_2$ or of $CrCl_2$, or 2×10^{-7} mole of $MnCl_2$ were injected in each of the groups. The 2×10^{-7} mole of $MnCl_2$ were injected, at the second arrow, to the $FeCl_2$ and $CrCl_2$ treated animals.

tween its parts. Thus we investigated the effects of some of these stable metals on the partition of the tracer amongst the organs of the body. The intracellular organelles could be also tested from that standpoint, particularly since it was observed earlier (13) that the mitochondria concentrate manganese isotopes. For this, one might have chosen an organ on the basis of some gross observation: As will be seen below, no such lead was forthcoming.

In view of the size of the enterprise involved in using all of the previously tested metals in this study as well, we had to arrive at a compromise: We chose elements 24, 25 and 26 (Cr^{++}, Mn^{++} and Fe^{++}).

For brevity, only Experiments A and B (Fig-

ure 5) are described out of four concordant ones (the others differed in several experimental details). In both of these the animals were divided into groups of three and eight, respectively, injected with radiomanganese tracer and tested for radioactivity on the first day. In Experiment A these groups were injected with 1×10^{-5} mole of sodium citrate or manganous, ferrous or chromous citrates per animals, respectively. The injections were made both in the morning and the evening of the second day. On the third, they were sacrificed within the hour following the test of their radioactivity. The ferrous citrate animals had given the impression of toxicity by displaying lesser physical activity and rougher furs than the controls. Thus, in Experiment B, the number of mice per group was increased, the manganous citrate group was omitted, the challenging dose of ferrous citrate was reduced to 2×10^{-6} mole and all the carriers were injected on the second and third days. The animals were sacrificed on the fourth day and were handled as in Experiment A.

The bodies of the animals were dissected and their organs divided as shown in Figure 5. The organs of each group were pooled and tested for radioactivity. Careful inspection of the peritoneal surfaces showed no metallic deposits.

As is shown in Figure 5, the only appreciable effect on the distribution of the tracer amongst the organs tested was again brought about by the manganese preparation. The others yielded a distribution comparable to that of the controls.

This phenomenon had the characteristics of the effect we had planned to show with the other stable metals, namely elution from one to another part of the body. It occurred above and beyond the accelerated elimination induced by manganese: In the experiments with the dissected organs, we have reported only the radioactivity retained in the body.

DISCUSSION

The uncertainty of projecting facts from the test tube into the realities of integrated life is clearly illustrated by this study. At the outset one was led to expect that several metals might elute radiomanganese from the body. Such expectation was based on the following facts: *1*) Many of the *in vitro* substitutes for manganese

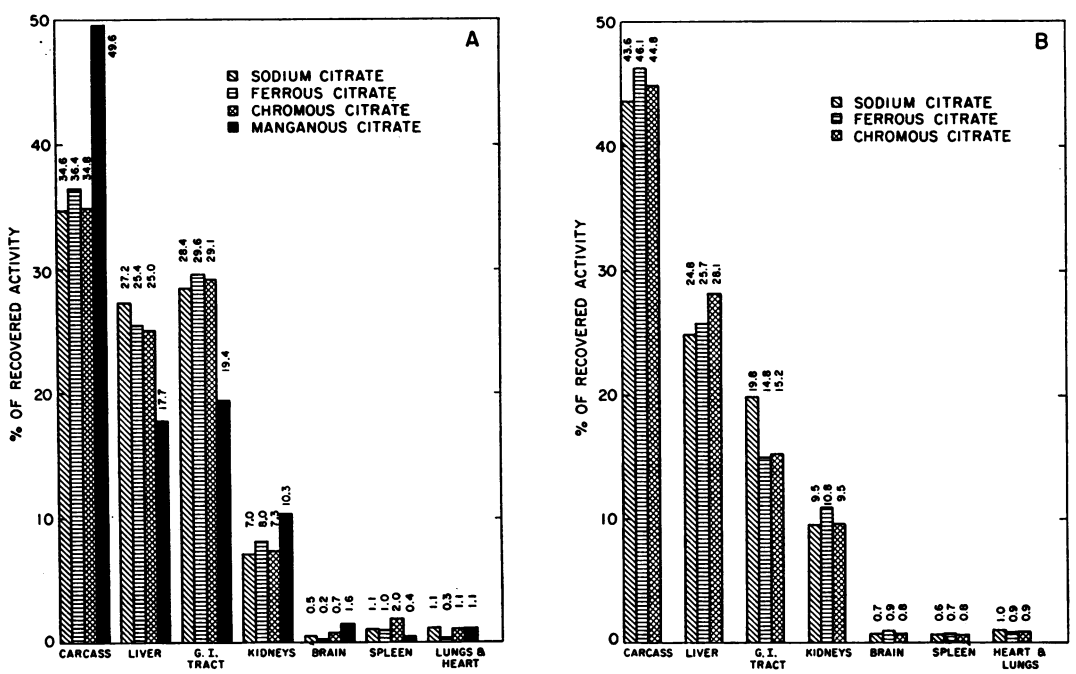

FIG. 5. PARTITION OF RADIOMANGANESE AMONG TISSUES OF MICE FOLLOWING INJECTION OF STABLE METAL COMPOUNDS

A and B summarize the results of Experiments A and B, respectively, described in the text.

bind more strongly with tissue constituents (2). *2*) Some are abundant, like magnesium, while the trace concentrations of manganese might not suffice to satisfy all its postulated reactions (14). *3*) The known *in vitro* transport mechanism does not guard against competitors (1).

A review of the coordination chemistry of manganese (2, 12) did not reveal any one single property which alone could explain these results. If *all* the known coordination properties are considered, however, this specificity can indeed be easily explained. Therefore, one is justified to assume that there exist in the body micro-compartments possessing many properties, the sum total of which determine the entrance of manganese and manganese only. These might include: a fairly resilient octahedral arrangement of six charges; oxygen as the predominating donor atom; a space with a radius of 0.5 to 0.9 Å. Such an environment would discriminate effectively against the entrance of other metals. If one also assumes that a given redox potential prevails at such a site, then specific reactions would be favored. A redox potential of minus 1.33 volts, for instance, would favor the reaction $Mn^{++} + H_2O = MnO_2 + 4H^+ + 2E^-$, while it would discriminate against other reactions.

The sum of such factors would constitute a specific segment in the anatomical pathway of manganese through the tissues of the body. The existence of specific atom configurations would not preclude binding of manganese by other less specific sites (2, 12). If this hypothesis were granted, one could ascribe many of the *in vitro* results to oversaturation of the few specific manganese sites with excess of metal. Such oversaturation would bring into evidence the properties of the numerous nonspecific sites. However, regardless of whether one favors this explanation, the observed irreplaceability of manganese in the body supports the concept of its performing specific tasks, rather than functions which might be taken over by other metals whenever the randomness inherent in the mass-law so dictates.

There is another extrapolation which one might have made *a priori* which also was shown by this work to be incorrect. One might have expected that manganese would be displaced by some metal (other than manganese) because of such well-known precedents as the displacement in the body of bromide by chloride (15), of molybdenum by tungsten (16) and of strontium by calcium (17). There exists, however, at least one metabolic difference between manganese and the elements listed above: the route of excretion. Manganese is excreted in the feces, while the others (Br, Mo, Sr, and so forth) are excreted primarily in the urine. This suggests that a search for other common differences might be rewarding. Furthermore it is obviously doubtful that manganese is the only substance whose pathway is strewn with specific segments, since one would expect similar behavior from iron also. With this in mind, an investigation of the pathways of other elements is now in progress.

SUMMARY

1. A procedure is described which permits observation of the elution of radiomanganese by injected stable metal compounds in intact mice and their tissues. This delineates an area for direct correlations between *in vivo* and *in vitro* phenomena.

2. Stable manganese compounds ranging from powdered metal to permanganate were effective eluting agents of the body's radiomanganese.

3. Injections of stable manganous citrate caused a pronounced change in the isotope's partition amongst the organs of these mice.

4. Stable members of the first transition group and of group VII of the elements as well as magnesium were tested for their capacity to elute the manganese tracer. In spite of injections of large doses, none of these affected the animal's normal rate of elimination of radiomanganese.

5. Chromous and ferrous citrates failed to cause deviations from the normal partition of Mn^{54} in the body.

6. The hypothesis is proposed that there exists a specific segment in the pathway of manganese through the body.

7. The specificity displayed by the manganese pathway is compared with the apparent lack of specificity illustrated by the *in vivo* displacement of bromide by chloride, of molybdenum by tungsten, of strontium by calcium and others. One common metabolic difference between these elements and manganese is noted: the route of excretion. This is entirely fecal for manganese and primarily renal for the other elements cited.

ACKNOWLEDGMENTS

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