Interdependence of routes excreting manganese

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The preceding papers show that the concentration of manganese in tissues is quite stable due to controlled excretion rather than to regulated absorption. Several excretory routes were found for this metal, all of which were linked to the gastrointestinal tract. Among these routes, the bile flow seemed to participate in an enterohepatic circulation of this essential element. Interruption of this circulation abolished or diminished the capacity of animals to accelerate the excretion of manganese after a metabolic load of this metal (3, 14, 18).

Methods

The rats, diets, housing, and isotopes used are described in the accompanying papers, as is the concentration of manganese in these diets (3, 14, 18).

Operations

All animals were studied shortly after laparotomy and while under ether anesthesia. Their bile ducts were catheterized over the uppermost third by means of no. 20 or no. 50 polyethylene tubing. The various segments of the gut were provided with in- and outgoing catheters constructed of no. 120 polyethylene tubing. The abdomen was covered with moist gauze.

Collections of Blood, Bile, and Intestinal Washings

Radioactive blood was collected from nicks in the tail veins into tared, heparinized capillary tubes. The radioactivity was measured and expressed per unit weight of whole blood.

Radioactive bile was routinely collected into tared, capped glass vials in which it was weighed and tested for radioactivity. After the initial phases of the biliary excretion were found to be exceedingly rapid the following collection schedule was adhered to: first four collections, 2.5 min; next four, 5 min; subsequent ones, 10 min. The bile was ascribed a specific gravity of 1 in converting weight to volume.

The intestinal segments were rinsed through every

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2.5 min with 1 ml normal saline solution. Four pooled rinses represented each 10-min collection.

The bile or plasma to be analyzed by neutron activation was collected with the precautions listed elsewhere (4a, 5, 7, 8, 17). Manganese 55 (stable) was measured together with the long-lived Mn64 (radioactive) as follows: on exiting the reactor the short-lived Mn66 (T1/2 = 2.58 hr) produced from the stable metal was measured together with the long-lived Mn64 (T1/2 = 314 days) since the photopeak used (840 kev) is common to both. After the passage of at least 24 hr the Mn64 was measured alone and Mn66 was quantified by difference. The standard error of the measurement by difference has not been assessed as yet, but as a rule the amount of Mn64 was less than that of Mn66.

Identification of Intestinal Excretory Routes for Manganese

In view of the above, the cephalad segments of the intestine were studied first in one Purina-fed animal. The bile duct was ligated and two intestinal segments were provided with ingoing and outgoing catheters, corresponding to the duodenum and the jejunum. They measured 5 cm in length and were located at a distance of 7 cm from each other. The rat was injected intravenously with 9 μc Mn64++ and the segments were rinsed as described above. The effluents from both contained identical amounts of radioactivity. These declined smoothly by more than one exponential rate over the 150 min of observation.

In another similar animal, excretion via the terminal ileum was compared to that of the duodenum and the jejunum. All three segments measured 3.5 cm. Again, the radioactivity excreted by the duodenum was not markedly higher than that excreted by the jejunum, although the former must have included the pancreatic juice. Figure 1 shows that the level of radioactivity discharged by these segments was initially 10 times higher in the case of the duodenum and the jejunum than in the case of the terminal ileum. Yet, the radioactivities from all three segments declined in parallel during the next 60 min of observation, suggesting that one might be measuring leached rather than excreted isotope. Therefore, the response of these segments was tested to the administration of manganese carrier (400 μg Mn64) calibrated doscs either with saline solution or with rat plasma as the vehicles. The latter was given in the form of MnSO4 parenterally, into the lumen of the exposed intestine, via the diet, or via the drinking water as indicated.

Ultrafiltrations were performed with the aid of a micro-method to be discussed elsewhere. Dialysis was performed with agitation in the cold after placing the bile in sacs constructed of viscose tubing. Sodium and potassium concentrations were generously measured by Mrs. L. Tassinari of this department, with the aid of flame photometry.

RESULTS

Three rats kept for 1 week on milk were given Mn64++ intravenously. Two were sacrificed 10 min later and one at 72 hr. The gastrointestinal tract from the pylorus to the ileocecal valve was stretched to about 110 cm and was cut into 1-cm segments which were assayed for radioactivity individually. The values were plotted as a function of distance from the pylorus. Regardless of the time elapsing after sacrifice, the most cephalad segment contained the highest amount of radioactivity. This declined smoothly as the distance from the pylorus increased. At a distance of 10–15 cm the concentration of the radioactivity declined to about 1/10 of the initial level and remained constant in all the more caudally located segments of the gut. Two Purina-fed animals showed a similar but less steep diminution of radioactivity.

Administration of Isotopes

The isotopes administered included the radioisotope Mn64++ (T1/2 = 314 days) discussed earlier (3, 14, 18) and the natural, stable Mn66++. The first was injected in...
identical in valence and injected in the same manner as the radioisotope (Mn⁴⁺). Figure 1 shows the rapid parallel increases of the radioactivity in the effluents from the duodenum and the jejunum as contrasted to the questionable response of the terminal ileum. This experiment was repeated twice on animals consuming Purina chow, whereas the comparison between duodenum and ileum was repeated once. In this latter case the smooth, exponential decline of the radioactivity in the respective effluents was followed for 110 min before the carrier was injected, but the response of the ileum, if present, was even less obvious than in Fig. 1.

Comparison Between Biliary and Intestinal Excretion

In one animal, also maintained on Purina chow, bile collections were performed together with washings of the duodenum after the intravenous injection of 9 μC Mn⁴⁺. Figure 2 shows the behavior of the radioactivity recovered from the respective routes. Surprisingly, the radioactivity appeared in the bile in the form of two distinct waves, in contrast to that in the duodenal washings which showed a continuous decline, again at more than one exponential rate. Furthermore, on iv injection of the 400 μg Mn⁴⁺, the output of Mn⁴⁺ kept slowly increasing in the bile, whereas a sharp increase occurred in the Mn⁴⁺ of the duodenal washings. Repetition of this experiment gave similar results.

Another experiment was identical in all details to the preceding ones except that the 400 μg Mn⁵⁺⁺ were given into the duodenum instead of intravenously. The jejunal effluents showed no change in the rate of decline of the Mn⁴⁺, whereas the second wave of the radioactivity in the bile showed a prolonged plateau, by sharp contrast to experiments like that on Fig. 1. Gross differences from these Purina-fed animals were encountered in two rats maintained on low-manganese milk for the 5 days preceding the experiment. In these latter instances, the original output of radioactivity in the bile declined faster than that in the jejunal washings and continued declining for the next 60 and 90 min, respectively. Indeed no second wave of radioactivity became evident in the bile of either animal. Introduction of 400 μg Mn⁴⁺ into the lumen of the duodenum was followed this time by an immediate sudden rise in the output of radioactivity in the bile only, which reached within 20 min a level 15 times higher than the pre-injection one. The jejunal output of radioactivity continued declining in both experiments.

The presence of unexpected components in the excretion curves of Mn⁴⁺ in the bile, their relatively fixed timing, and the apparent reciprocity between biliary and intestinal excretion, led to detailed study of the excretion of manganese in bile. These experiments are presented as follows: 1) comparisons are drawn between the biliary excretion of intravenously injected tracer and its blood clearance; 2) contrasts are drawn between conditions which failed to affect the tracer's excretion pattern and those which permitted imposition of selective changes at will; 3) experiments are presented indicating that this pattern indeed reflects the metabolism of this essential metal rather than simply that of the tracer used here arbitrarily.

Time Sequences in Excretion of Mn⁴⁺ Into the Bile

An effort was now made to sample the bile rapidly by adhering to the schedule discussed under METHODS unless indicated otherwise.

The sequence shown in Fig. 3 was encountered in the 10 Purina-fed rats injected with tracer as soon as the bile flow was exteriorized. The radioactivity increased sharply in the bile to reach a peak 5-7 min after injection of the tracer. A rapid decline followed thereafter and a plateau was reached about 20-30 min after injection of Mn⁴⁺, which lasted for about 30 min. When the radioactivity was measured concomitantly in blood (sampled from the vena cava) and the bile, the blood clearance curve preceded by a few minutes the first wave of radioactivity in the bile. However, the concentration of the tracer was 10 times higher in the bile than in the corresponding blood samples. Approximately 40 min after the injection of the tracer the two curves became totally dissociated; while the blood-bound isotope continued on its decline the biliary concentration began to increase.

In conformity with the results shown in Fig. 3, all Purina-fed animals tested showed two waves in the concentration of the radioactivity in the bile. The first one reached a peak 7.5-10 min after the injection of the tracer, depending on the length of the catheter. The second wave reached a peak 60-120 min after the tracer's injection, followed immediately by a decline to the levels of the plateau encountered between the first and second waves. Extension of two experiments to approximately 8 hr revealed no third wave.

These findings made it necessary that the blood
clearance be defined in the rat as it had in man (2). The data shown in Fig. 4 were normalized at the 6-min point.

As expected, the half-life of the initial components of the blood clearance curve was less than 1 min. The rapidity of sampling displayed in Fig. 4 could not be duplicated with bile collections without administering prohibitive amounts of radioactivity. Therefore only a gross intercomparison between the blood clearances and the excretion curves could be made. For this, data from all animals discussed in this paper and obtained shortly after injection of the tracer were plotted as “cumulative excretion.” Over the first 6 min this showed a doubling time of somewhat less than 1 min. As an illustration, Fig. 5 represents animals consuming a) milk; b) a commercial low-manganese diet (13); and c) this diet plus manganous sulfate in the drinking water. It is evident that the cumulative excretion did not reflect the dietary supply of this metal over the first 6 min. The latter parts of these curves, however, seemed to reflect the level of dietary manganese, but only the duration, not the height, of the second wave was affected by feeding massive amounts of metal.

Conditions That Did Not Affect Biliary Excretion Pattern of Mn\textsuperscript{54}

The initial bile flow in these experiments varied between 6 and 12 mg/min and declined slowly thereafter. Both waves of radioactivity seemed independent of the bile flow, regardless of whether the tracer was dissolved in rat serum or in saline. The following variables, evaluated for their effects on these two waves gave negative results. In one rat, distending the bile duct was avoided by entering the unclamped duct; in one the portal instead of a peripheral vein was used for the injection of the isotope. In one there was injected 0.5 ml 0.9 % NaCl solution into the duodenal lumen every 10-20 min; in three, the saline solution was given subcutaneously; in another, the bile samples were injected into the duodenum after their weight and radioactivity were determined. In others, the adrenal glands or the thyroid and parathyroids were removed prior to the injection of the isotope. Two Wistar strain rats instead of Sprague-Dawley rats were tested.

Conditions That Affected Biliary Excretion Pattern of Mn\textsuperscript{54} Selectively

Changes of the first wave. The previous experiments suggested that the first wave might reflect, at least in part, the initial events of the blood clearance of injected Mn\textsuperscript{54}. This was supported by experiments in which the bile flow was exteriorized after sufficient time had elapsed for the injected isotope to reach negligible levels in the blood stream (Fig. 4). Five Purina-fed rats were studied 16 hr to 5 days after the injection of Mn\textsuperscript{54}. In all of these the initial decline of radioactivity was very small if present at all, but the second wave was invariably present; after about 30–50 min the concentration of Mn\textsuperscript{54} in the bile samples began to increase sharply and reached levels between 10 and 35 times higher than the level present at the beginning of these waves. Yet, neither the height of this second wave nor its duration could be correlated with the time which had elapsed between injection and observation.

Changes of the second wave. Since injecting the isotope before the initiation of the bile collections affected only the first wave of Mn\textsuperscript{54}, it was thought that injecting the isotope after the bile had been allowed to flow freely might affect only the second wave. The bile was permitted to flow in two freshly operated rats for 3 hr preceding the injection of the isotope. The first wave of radioactivity was present but the second was absent in both animals.

The experiment suggested that the second wave might signal the discharge of stable, natural manganese into the liver from some unidentified source. To test the soundness of this assumption, four rats were injected with Mn\textsuperscript{54} 24 hr before canulation of the duct and their bile was permitted to flow freely for about 3 hr. After the initial concentrations of Mn\textsuperscript{54} in bile were established, these animals were injected intravenously with 0.02, 0.1, or 2.0 mg nonradioactive manganese. The injection of the stable metal was followed by a rapid increase of the concentration of the radioactive isotope in the bile.
This was roughly proportional to the amount of carrier metal injected, as shown primarily by the duration of the ensuing waves of radioactivity. In one of these animals, the radioactivity was measured in the blood together with the bile. Both pools showed an increase in the isotope's concentration following administration of 2.0 mg manganous sulfate, but the increase amounted to only 25% in the blood while a 10-fold level was reached in the bile. Furthermore the ensuing waves lasted for 20 vs. 80 min, respectively.

In view of the above, it was thought that depleting the intestinal contents of manganese would abolish this wave. Three rats were fed the low-manganese diet for 14 days and received injections of Mn\textsuperscript{44} as soon as the catheter was inserted. The first wave was clearly evident in the bile samples of all these animals but the second wave was absent in all.

These data suggested that the second wave of radioactivity in the bile indicated dietary manganese being absorbed from the gut. Confirmatory evidence was gathered in two experiments generously performed by Dr. G. Gerber of the Euratom Laboratory, Brussels, Belgium. In these the perfused-liver technique was used in vitro. Injection of Mn\textsuperscript{44} into the arterial side of the perfusion system was followed by an early, single wave of the radioactivity in the bile, regardless of whether the donor animals had been kept on milk or on milk plus 0.005 M Mn\textsuperscript{44}. Plotting these data as cumulative excretion revealed a double time of somewhat less than 1 min, as in Fig. 5.

If the second wave of the radioactivity in the bile was indeed linked to the absorption of the dietary manganese, the concentration of the natural Mn\textsuperscript{44} should vary in a manner similar to the tracer. This was investigated in the experiments which follow.

**Biliary Excretion of Mn\textsuperscript{44}**

Duplicate analyses of the serum manganese (Mn\textsuperscript{44}) concentration were performed on three Purina-fed rats and three animals maintained on milk for about 2 weeks. The respective mean serum concentrations (and ranges) were: 3.19 (3.00-3.32) µg/liter vs. 3.10 (2.70-3.24) µg/liter. These concentrations markedly contrasted to our previously published values for rat bile, which were both higher and much more variable (17). This suggested that the concentration of natural manganese in bile might also depend on the time which had elapsed between catheterization and sampling. Therefore the sequential analyses for Mn\textsuperscript{44} and Mn\textsuperscript{44} were performed.

In two animals reared on the Purina diet, the bile duct was entered and the radioisotope was injected intravenously. Bile collected over 10-min periods was analyzed both for the natural metal and for the radioisotope. The results were the same in both experiments; Fig. 6 shows the sequences found in one of them. The initial concentration of the natural isotope (Mn\textsuperscript{44}) in the bile was about 20 µg/liter. This declined together with the concentration of the radioisotope but not as rapidly. About 50 min after initiation of these experiments the customary second wave was again present and now it involved both the stable and radioactive isotopes.
Still the specific activity of the radioactivity declined gradually with a half-life of 60-70 min, proving that the tracer was tracing. This experiment was repeated on an animal maintained for only 3 days on the milk diet (Fig. 7). The initial concentration of the stable manganese isotope was 12 μg/liter and slowly doubled itself over the next 50 min. At that time 1 mg Mn^{66+} (as MnSO_4) in 1 ml water was injected into the duodenal lumen. An immediate sudden rise of the manganese concentration in the bile occurred, which reached 27.5 ± 1.6 mg/liter at the end of 50 min (Fig. 7). The total output of Mn^{55} in the bile during this observation amounted to only 0.3% of the dose injected into the duodenum.

Since the concentrations of manganese showed a sharp gradient between plasma and bile, a comparison was made with the respective concentrations of sodium and potassium in both pools. The plasma concentrations of these alkali metals in our rats have ranged, respectively, between 135 and 145 mEq/liter for sodium and 4.0-5.0 mEq/liter for potassium. In three rats the sodium concentration in bile ranged between 162 and 167 mEq/liter, whereas the potassium concentration ranged between 5.3 and 5.4 mEq/liter. These concentrations did not change with passing time.

State of Mn^{55} in Bile

The presence of these two waves suggested the existence of two states of the metal in bile. Hence several tests were performed in vitro on representative samples. In four electrophoretic experiments, the mobility of the bile-bound isotope was identical to that of an aqueous solution of carrier-free Mn^{55} used as control, whether the bile had been tagged in vitro or in vivo. No significant differences could be elicted between bile samples collected immediately or 2 hr after the injection of the Mn^{55} and ascorbic acid. However, in one bile sample collected during the preponderance of the second wave, there was encountered in addition a negatively charged component. This was noteworthy because it had the same electrophoretic mobility as the β-globulin of serum, known to contain a metal-binding fraction.

Ultrafiltration of bile samples collected during the first and second waves showed that 16% of the total isotope was ultrafilterable in both. Dialysis of such samples against triple-distilled water showed the radioactivity of bile to be fully dialyzable, whereas only about 4% of the radioactivity in plasma was ultrafilterable. The only clear-cut, reproducible difference was encountered when bile from Purina-fed rats was compared to that from similar animals that had been injected with 1 mg MnCl_2. After freezing overnight and thawing, the samples obtained from the injected rats showed a gross precipitate which was fine in texture and grayish in color. This precipitate contained 90% of the Mn^{55} present in bile and was visible only after freezing and only after injection of this manganous salt. Its nature has not been investigated.

DISCUSSION

The excretion of manganese via several routes was suggested by other communications (4, 12, 18) indicating possible similarities with the excretion of zinc (6). The present work focused on the cephalad segments of the intestine, and the tributaries emptying therein, as the significant excretory routes. This localization seems provocative in view of the common embryological origin of these structures from the primordial gut (18). It would be intriguing if the similarities demonstrated among these routes prove to reflect common origin but the differences prove linked to differentiation. Indeed, the similarities were restricted primarily to the shapes of the respective excretory curves, whereas the differences were several and worthy of discussion.
The various curves representing excretion of manganese from the gut showed that much higher amounts of tracer were excreted by the duodenum and jejunum than by the terminal ileum. Furthermore, the cephalad segments responded briskly to manganese loading whereas the terminal ileum did not, suggesting that the cephalad segments might be homeostatic end organs auxiliary to the liver.

Animals reared on a standard laboratory diet excreted the radioisotope into the bile in the form of two distinct waves. The first of these appeared to be linked to the blood clearance of Mn\(^{34}\), and must have signaled a direct passage of tracer from the plasma into the bile (Figs. 3-5). The manganese thus transferred did not constitute a major portion of the metal in bile, since there was found a steep gradient between plasma and bile. This sharp gradient was evident regardless of whether the radioisotope, Mn\(^{34}\), or the stable isotope, Mn\(^{55}\), were quantified. This gradient was much steeper in the case of manganese than in the case of either sodium or potassium, suggesting a different transport mechanism from those of alkali metals (21).

This gradient became even steeper during the preponderance of the second wave. It appears that this wave signaled that exteriorization of the bile flow had set off a marked acceleration of the enterohepatic circulation of this metal. The existence of such a circulation was proposed earlier (18), and was directly documented here (Fig. 7). That the second wave reflected an acceleration of this circulation is summarized on the basis of the following evidence: 1) This wave was present when animals were consuming a high-manganese diet.

2) It was absent when the animals ate a low-manganese diet for several days, when it was most unlikely (3, 14) that they could have developed manganese deficiency.

3) In Purina-fed animals, manganese loads increased the duration of this wave. 4) In milk-fed animals this wave was induced readily by manganese loads, given enterally or parenterally. 5) The bile from isolated, perfused livers did not show this second wave. 6) No second wave occurred spontaneously in the intestinal effluents but it could be induced here also, by means of loading with manganese.

The experiments in which the bile and intestinal effluents were analyzed concurrently showed the following: animals consuming a high-manganese diet (Purina) responded to parenteral manganese loads primarily by producing a radiation wave in the intestinal effluents, but not by discernible increases in the height of the second wave in the bile (Fig. 2). By contrast, animals consuming a low-manganese diet (milk) seemed to respond by producing a second wave of the radioactivity in the bile but not in the jejunal effluents. Hence it appears that, when the excretion by the liver became saturated, auxiliary excretion occurred via the gut. This seems to explain the bypass of the liver encountered earlier (18).

The mechanism by which this set of events was brought about remains obscure. It must be re-emphasized that these animals were freshly laparotomized and under ether anesthesia. This circumstance does not jeopardize the validity of comparisons made on the same animal but it detracts seriously from extrapolations to intact animals (13, 14, 18). It is highly desirable that surgery and observation be temporally separated in future experiments for the following reasons: Another set of essential nutrients, the bile acids, are known to undergo enterohepatic circulation in a manner similar to manganese. Exteriorization of the bile flow has induced fluctuations of the bile acid concentration similar to, but out of phase with, the fluctuations of the manganese concentration encountered here (11, 15, 20). In the rat, the bile acids are formed directly from cholesterol and their enterohepatic circulation acts as a feedback control mechanism inhibiting cholesterol synthesis (16). In this context, it is most intriguing that manganese is established as an important cofactor in cholesterol synthesis (1, 9, 10, 19). Hence it is possible that the unusual excretory pattern of manganese in the bile may have physiological implications transcending the laparotomized, anesthetized animals studied here.

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REFERENCES


