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# Measurement of Thrombocytopoiesis in W/W$^v$ Mice with Evidence for an Abnormality of Sulfate Metabolism[1] (41218)

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*Abstract.* Mice of the W/W$^v$ genotype have an hereditary macrocytic anemia resulting from a diminished proliferative capacity of hemopoietic precursor cells. Platelet production was studied because W/W$^v$ mice maintain a normal number of circulating platelets that are of normal size in spite of a diminished number of megakaryocytes. Using [$^{75}$Se]selenomethionine and calculating the percentage of the injected dose incorporated by platelets, the platelet production rate of W/W$^v$ mice was found to be equivalent to the production rate of their +/+ littermates. Using $Na_2{}^{35}SO_4$, however, the platelet production rate, as measured by the same isotope incorporation method, was nearly twice the rate of the normal littermates. The increase in $^{35}$S incorporation appeared to be explained by a greater availability of isotope in the W/W$^v$ as evidenced by the higher plasma levels of $^{35}$S that were concurrently found. This suggests that the W/W$^v$ mouse has an aberrant metabolism of inorganic sulfate probably unrelated to platelet production. These results also demonstrate how failure to take plasma radioactivity levels into account can lead to conflicting and probably erroneous conclusions in animals with an inherent or induced metabolic abnormality.

Mice of the W/W$^v$ genotype have a hereditary macrocytic anemia that appears to be due to an abnormal hematopoietic precursor cell and is correctable by bone marrow transplantation from a normal donor (1). In addition to the macrocytic anemia, other demonstrated hematopoietic abnormalities include decreased granulocytes and megakaryocytes in the bone marrow and spleen (2), and the failure of W/W$^v$ stem cells to proliferate and differentiate properly after transplantation (3). The diminished numbers of megakaryocytes appear to be capable of maintaining normal platelet production as evidenced by the normal count and size of platelets in W/W$^v$ mice (4). However, it could be suggested that W/W$^v$ platelets may have a prolonged survival to account for the normal numbers of platelets in the presence of a substantial reduction in megakaryocytes. Therefore, the present experiments were done to compare the rate of platelet production in W/W$^v$ mice and their normal +/+ littermates.

$Na_2{}^{35}SO_4$ and [$^{75}$Se]selenomethionine have been used to measure relative rates of platelet production in normal, stimulated, and suppressed animals, as well as to quantify the thrombopoietic activity of injected substances (5–8). These radioisotopes are incorporated into megakaryocyte cytoplasm, and the percentage of the injected dose of radioisotope that is incorporated into subsequently released platelets is determined as a measure of platelet production. Various methodologies have been used in making this determination but in all methods the percentage incorporation into platelets is calculated with the formula,

$$\text{Percentage isotope incorporation} = \frac{\text{total platelet radioactivity}}{\text{radioactivity of injected dose}} \times 100.$$

Plasma radioactivities are generally not considered in the percentage incorporation studies except in the technique of Evatt *et*

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*al.*, in which they correct for plasma contamination of the platelets (7, 8). This report will demonstrate the importance of monitoring plasma radioactivity as a measure of metabolism and availability of the tracer substance. Failure to take plasma radioactivities into account would have led to conflicting and probably erroneous conclusions in W/W$^v$ mice.

**Materials and Methods.** Female WBB6F$_1$/J-WW$^v$ mice and their normal +/+ female littermates were obtained from the Jackson Laboratory, Bar Harbor, Maine, and used at 12–14 weeks of age. $Na_2{}^{35}SO_4$ was obtained from New England Nuclear, Boston, Massachusetts in a concentration of 2 mCi/ml and specific activity of 850 mCi/mmole. [$^{75}$Se]selenomethionine was obtained from E. R. Squibb & Sons, Inc., Princeton, New Jersey, in a concentration of 200 $\mu$Ci/ml and a specific activity of 162 mCi/mg.

Each mouse in the $Na_2{}^{35}SO_4$ studies received 25 $\mu$Ci intraperitoneally and each mouse in the [$^{75}$Se]selenomethionine study received 2 $\mu$Ci. At 24, 48, and 72 hr after isotope injection, 0.5 to 1.0 ml of blood was drawn by cardiac puncture under ether anesthesia and anticoagulated with dry $K_2$ EDTA. A platelet count was done by phase microscopy (9) and a hematocrit with a Drummond microhematocrit machine.

The blood sample was then centrifuged at 1100*g* for 30 min and the platelet-poor plasma (PPP) removed and saved. The blood cells were resuspended with 1 ml saline and centrifuged at 120*g* for 15 min. Platelet-rich plasma (PRP) was removed, its volume was measured, and its platelets counted by phase microscopy. The platelet suspension was then washed once by adding 4 ml of 1.0% ammonium oxalate, mixing, and centrifuging at 1100*g* for 30 min. The platelet button was then washed twice with 4 ml saline.

For $^{35}$S measurements the platelet buttons were digested with 2 ml of NCS (Amersham/Searle, Arlington Heights, Ill.), 15 ml of scintillation solution was added, and counts were done in a Packard liquid scintillation counter. For $^{75}$Se measurements, the buttons were suspended in 1 ml $H_2O$ and counted in a Nuclear Chicago automatic gamma counter. For both isotopes, 0.1 ml of PPP was counted in the same manner as the platelet button. Injected radioactivity was measured by counting a 1:1000 dilution aliquot of the injected material.

Total platelet radioactivity was calculated for each sample as follows: cpm/platelet × total circulating platelets, where cpm/platelet equaled cpm/button ÷ (platelet count PRP × volume of PRP), and total circulating platelets equaled whole blood platelet count per milliliter × 0.06 × body weight in grams.

**Results.** Table I displays the weights and blood counts of the W/W$^v$ and +/+ mice that were used in these experiments. All of the weights were comparable and the platelet counts were normal. The W/W$^v$ mice differed from the +/+ mice in having the expected level of anemia.

Percentage isotope incorporation was determined in the standard manner by dividing the calculated total platelet radioactivity by the radioactivity of the injected dose. The results (Fig. 1) show that the percentage incorporation of $^{35}$S was increased

TABLE I. WEIGHTS AND BLOOD COUNTS OF W/W$^v$ AND +/+ MICE

| | *n* | Hematocrit (%) | Weights (g) | Platelets/mm$^3$ ($\times 10^{-6}$) |
|---|---|---|---|---|
| | | $Na_2{}^{35}SO_4$ | | |
| W/W$^v$ | 26 | 36.2 ± .4[a] | 22.1 ± .4 | 1.63 ± .06 |
| +/+ | 26 | 45.6 ± .5 | 21.8 ± 1.5 | 1.47 ± .17 |
| | | [$^{75}$Se]Selenomethionine | | |
| W/W$^v$ | 31 | 35.1 ± .9 | 24.1 ± .8 | 1.54 ± .14 |
| +/+ | 31 | 45.6 ± .9 | 23.2 ± .4 | 1.42 ± .04 |

[a] Each value equals mean ± SEM.

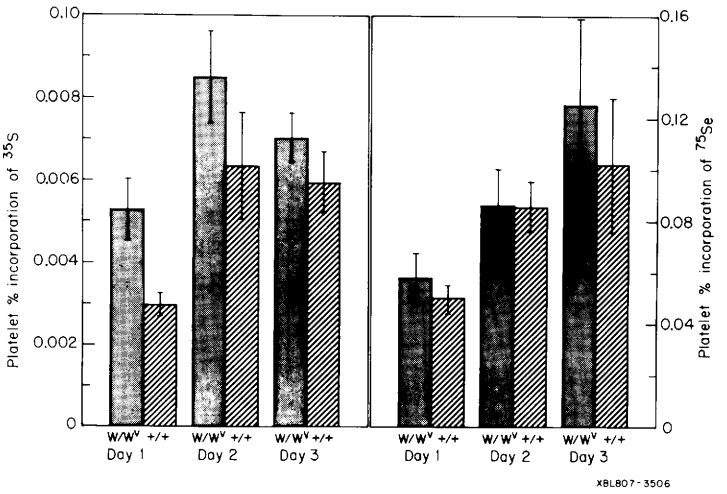


FIG. 1. Comparison of W/W$^v$ and +/+ percentage incorporation of $^{35}S$ and $^{75}Se$ into platelets. Results represent the means ± SEM of two experiments for each isotope measured at 24, 48, and 72 hr after isotope injection. Each point represents 10 or 11 mice except for $^{35}S$ on Day 3 which represents one experiment with 5 mice.

in W/W$^v$ mice over that of the +/+ controls by 77, 34, and 20% at 24, 48, and 72 hr, respectively. The 24-hr difference was significant at the $P < 0.01$ level, and the 48-hr at $P < 0.2$. However, the percentage incorporation of $^{75}Se$ was comparable in W/W$^v$ and +/+ mice, and no significant difference could be demonstrated at these same time points. We then compared the plasma levels of radioactivity in the test animals as shown in Fig. 2. When $Na_2{}^{35}SO_4$ was used as the tracer isotope, the plasma radioactivity levels were also significantly higher in W/W$^v$ mice than in the +/+ littermates, while with [$^{75}Se$]selenomethionine the plasma radioactivities were equivalent. To test whether the increases in platelet radioactivity were proportional to those of the plasma, the data from each test mouse were recalculated using the ratio:

$$\frac{\text{total platelet radioactivity}}{\text{radioactivity of 0.1 ml of plasma}}.$$

Figure 3 shows that the relationship of the platelet to plasma radioactivity was the

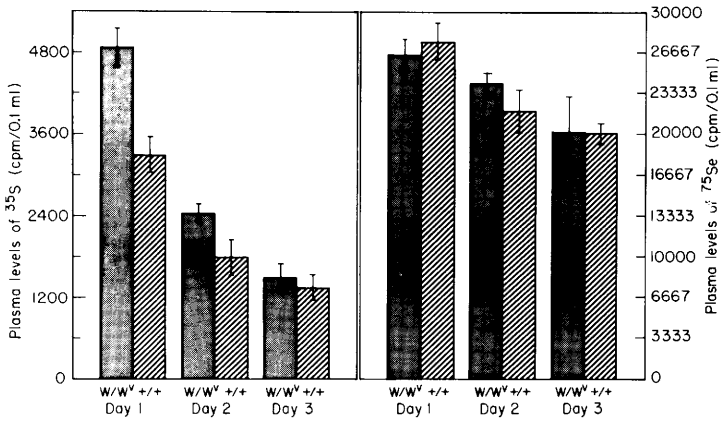


FIG. 2. Comparison of W/W$^v$ and +/+ levels of isotope incorporated into 0.1 ml of plasma. Sample point details are the same as for Fig. 1.

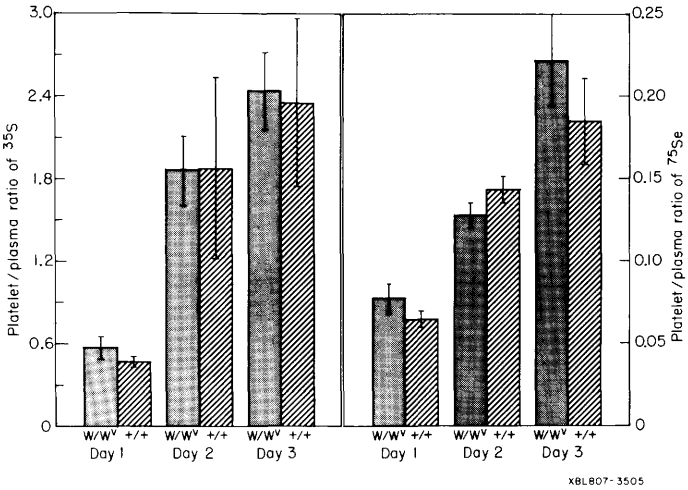


FIG. 3. Platelet-to-plasma ratio of incorporated isotope. Sample points are the means ± SEM of the data given in Figs. 1 and 2, individually calculated for each mouse.

same in both W/W$^v$ and +/+ mice at each time of sampling. Although values for the ratio differed with the two different isotopes, the results were comparable.

**Discussion.** The use of radioactive tracers to measure platelet production requires that megakaryocytic uptake of the tracer substance be proportional to the cytoplasmic volume of the platelets that are produced in the presence of available isotope. Previous publications indicate that both $Na_2{}^{35}SO_4$ and [$^{75}$Se]selenomethionine meet this requirement (5–7). In addition, when isotope incorporation is used to compare groups of animals, it is necessary to assume that isotope availability is the same in test and control animals when they receive the same administered dose of tracer. Clearly, any differences in isotope distribution, elimination, or alternate pathway metabolism will invalidate this assumption.

When W/W$^v$ and +/+ mice were compared using both $Na_2{}^{35}SO_4$ and [$^{75}$Se]selenomethionine, conflicting results were obtained. With $Na_2{}^{35}SO_4$ as the tracer substance, the W/W$^v$ appeared to have an increased rate of platelet production when measured by percentage isotope incorporation, but with [$^{75}$Se]selenomethionine the platelet production was normal. One hypothesis that might resolve this conflict would be that W/W$^v$ megakaryocytes have an abnormal metabolism which causes them to take up excess sulfur in an equivalent volume of megakaryocyte cytoplasm. However, since the plasma radioactivity was proportionally increased in the W/W$^v$, it is more likely that some abnormality in either distribution, elimination, or alternate pathway metabolism, unrelated to platelet production, altered the size of the isotope pool available for synthesis of both megakaryocyte cytoplasm and plasma components. Dassin and Najean (10) have reported that sulfate is incorporated into platelet-sulfated mucopolysaccharides with virtually none of the radioactive sulfur detectable in the papain-digested platelet protein fraction. [$^{75}$Se]Selenomethionine, in which $^{75}$Se is substituted for the methionine sulfur, is metabolized as an amino acid and incorporated predominantly into platelet and plasma proteins (10, 11). Since the metabolic products of these two sulfur-containing substrates are not the same, it is likely that availability of each to metabolic pathways is separately regulated.

Further evidence of abnormal sulfate metabolism in W/W$^v$ mice has been reported by McCuskey and Meineke who, by histochemical staining, have demonstrated different abnormal distributions of sulfated acid mucopolysaccharides in the hemopoietic organs of W/W$^v$ and S1/S1$^d$ mice

(12). We have reanalyzed previously published $S1/S1^d$ data (13) and found that its abnormal distribution does not appear to significantly affect isotope availability in the $S1/S1^d$, as evidenced by the equivalent plasma isotope levels compared to controls and comparable results using either the percentage incorporation or platelet to plasma calculations.

These results show that platelet production is normal in $W/W^v$ mice and provide evidence that these mice have an inherent abnormality of metabolism affecting sulfate ions. They further indicate the importance of monitoring plasma levels of a radioisotopic label, possibly as an independent measure of isotope availability, to validate corresponding measurements of platelet incorporation of the tracer substance.

1. Harrison, D. E., and Russell, E. S., Brit. J. Haematol. **22**, 155 (1972).
2. Chervenick, P. A., and Boggs, D. R., J. Cell. Physiol. **73**, 25 (1969).
3. McCulloch, E. A., Siminovitch, L., and Till, J. E., Science **144**, 844 (1964).
4. Ebbe, S., and Phalen, E., J. Cell. Physiol. **96**, 73 (1978).
5. Odell, T. T., Jackson, C. W., and Reiter, R. S., Acta Haematol. **38**, 34 (1967).
6. Harker, L. A., J. Clin. Invest. **47**, 458 (1968).
7. Evatt, B. L., and Levin, J., J. Clin. Invest. **48**, 1615 (1969).
8. Evatt, B. L., Shreiner, D. P., and Levin, J., J. Lab. Clin. Med. **83**, 364 (1974).
9. Brecher, G., and Cronkite, E. P., J. Appl. Physiol. **3**, 365 (1950).
10. Dassin, E., and Najean, Y., Acta Haematol. **61**, 61 (1979).
11. Lathrop, K. A., Johnston, R. E., Blau, M., and Rothschild, E. O., J. Nucl. Med. Suppl. No. 6 MIRD Pamphlet No. 9, 10 (1972).
12. McCuskey, R. S., and Meineke, H. A., Amer. J. Anat. **137**, 187 (1973).
13. Ebbe, S., Phalen, E., D'Amore, P., and Howard, D., Exp. Hematol. **6**, 201 (1978).

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