# Incorporation of Labeled Glycine into Erythrocyte Glutathione of Rabbits; Effect of Nutritional Muscular Dystrophy.* (27946)

J. F. Diehl and W. M. Riddell
*Department of Biochemistry, University of Arkansas Medical Center, Little Rock*

Several authors have reported increased glutathione (GSH) levels in muscle and liver of dystrophic rabbits raised on a Vit. E-deficient diet(1,2,3). It has been suggested that the metabolism of GSH is to some degree controlled by Vit. E(3). Ryerson *et al.* have found erythrocyte GSH in Vit. E-deficient rabbits to be approximately 33% higher than in control rabbits(3). This finding was of particular interest because of the known sensitivity of the erythrocytes of Vit. E-deficient animals to hemolysis(4) and because of the disputed relationship of erythrocyte glutathione content to hemolysis(5). Red blood cell GSH is in a dynamic state and its metabolism has been studied *in vitro* and *in vivo* with labeled precursors(6,7,8). The present report is concerned with the effect of nutritional muscular dystrophy on rate of incorporation of glycine-1-$C^{14}$ into red blood cell GSH *in vivo*.

*Methods.* Nutritional muscular dystrophy was produced in White New Zealand rabbits of mixed sex. The animals were approximately 4 weeks old when they were placed on a semi-synthetic diet consisting of 29.5 g sucrose, 27 g corn starch, 25 g cellulose (Alphacel), 11.3 g casein ("vitamin-free"; Nutritional Biochemicals Corp.), 2.25 g stripped lard (Distillation Products Ind.), 2.25 g cod liver oil, 2.25 g H.M.W. salt mix (Nutritional Biochem. Corp.), 375 mg vitamin mix,† 75 mg inositol, 75 mg choline chloride, 3.5 μg Vit. $B_{12}$. This is essentially the Vit. E-deficient diet described by Young and Dinning(9) with cellulose added as roughage and the mixture fed in the form of cookie-sized pieces as previously described (10). This diet produces muscular dystrophy in young rabbits within 3 to 4 weeks. The animals die a few days after first signs of muscular weakness appear, unless they are treated with Vit. E supplements. A single large dose of α-tocopherol reverses metabolic symptoms of Vit. E-deficiency such as creatinuria and increased $CO_2$ production within one or 2 days(11) and repeated supplements of α-tocopherol restore muscular strength in about one week. Animals receiving the basal diet in the present study gained approximately 8 g per day during the first 2 to 3 weeks. Body weight then remained constant or fell during the terminal stage of the deficiency disease. Animals designated as controls received the basal diet with oral supplements of 12 mg α-tocopherol acetate per kilogram body weight 3 times weekly. Average weight gain in this group was 11 g per day during the whole experimental period.‡ To minimize the effect of possible coccidiosis infection on their Vit. E status(12), all animals received 0.04% sodium sulfaquinoxaline in the drinking water for 3-day periods alternating with 2-day periods of plain water.

Animals in the experimental group were injected intraperitoneally with 10 μc of glycine-1-$C^{14}$ per 100 g of body weight (specific activity 5 mc per mmole) when they showed symptoms of advanced muscular dystrophy. Animals in the control group were injected after they had been on the experimental diet for approximately 3 weeks. Blood was drawn from different animals by heart puncture with a heparinized syringe at 0.5, 1, 2, 3, 4, and 12 hours after injection. Immediately after the heart-puncture the rabbits were sacrificed for other studies such as the determination of skeletal muscle protein radioactivity previously described(13). Red blood cells and plasma were separated by centrifugation. Plasma was pipetted off and

* Supported by a research grant from Nat. Inst. Health, U. S. Public Health Service.

† Vitamin mix: nicotinamide 4 g, pyridoxine 100 mg, thiamine chloride 100 mg, riboflavin 100 mg, calcium pantothenate 200 mg, folic acid 100 mg, biotin 1 mg, menadione 5 mg, casein to make 100 g.

‡ Rabbits of the same stock and age gain approximately 30 g per day on commercial rabbit chow.

stored frozen for later determination of glycine specific activity (s.a.). The buffy coat was removed and the red cells were immediately used for determination of GSH radioactivity.

Glutathione was isolated as the cadmium salt and purified by reprecipitation as the copper mercaptide by the method of Waelsch and Rittenberg(14), using 0.4 ml of packed erythrocytes and 25 mg of non-radioactive glutathione as carrier. When enough blood was available the isolation procedure was run with four 0.4 ml portions of erythrocytes. With less blood available the isolation was run in triplicate or duplicate. The copper mercaptide thus isolated was suspended in ethanol and placed in planchets. After drying under a heat lamp the samples were counted with a Tracerlab gas-flow Geiger tube. Counts were corrected for background and extrapolated to infinite thinness.

GSH concentration was determined by the alloxan 305 method of Lazarow(15) using 1 ml samples of whole blood. The hematocrit value was used to calculate the GSH concentration per ml of packed erythrocytes. This is permissible because all the GSH of blood is in the red cells(16). Determination of GSH concentration and isolation of the mercaptide had to be carried out on fresh blood in order to avoid loss of GSH. Two workers were required to carry out these procedures. In some cases (for instance when rabbits became dystrophic on weekends) both workers were not available and in such instances mercaptide isolation alone, without parallel GSH determination, was carried out.

Glycine was isolated by a combination of paper electrophoresis and paper chromatography. Two milliliters of plasma (or whole blood) were treated with 2 ml of 10% trichloroacetic acid and centrifuged. A 1 ml aliquot of the supernatant was taken to dryness *in vacuo* and the residue was transferred with a few drops of water to a paper strip. Electrophoresis was carried out under previously published conditions(17). After 4 hours the strips were removed from the electrophoresis cell, and dried. The area known to contain the neutral amino acids was cut out and extracted with water. Eluates thus obtained were concentrated *in vacuo* and transferred to paper sheets for descending chromatography with an 80:20 pyridine: water mixture(18). A 15- to 18-hour run separated glycine from other neutral amino acids. The glycine location was ascertained by ninhydrin development of a simultaneously run glycine sample. Glycine containing areas were cut out and eluted. Half the volume of the eluate was used for reaction with the ninhydrin reagent of Rosen(19), reading the color density against a blank prepared by eluting a comparable area of filter paper which was taken from a section of the chromatogram known to be free of amino acids. Solutions of known glycine concentration were used to obtain standard curves. The other half of each eluate of radioactive glycine was evaporated on a planchet and counted under the Geiger tube. Specific activity of plasma (or whole blood) glycine was calculated from the data thus obtained.

TABLE I. Specific Activity (± Standard Deviation) of Erythrocyte Glutathione Isolated with Carrier Glutathione. No. of animals in parentheses.

| Time after inj, hr | Control rabbits, cpm/mg | Vitamin E-deficient rabbits, cpm/mg |
|---|---|---|
| .5 | 12.0 ± 2.8 (3) | 14.4 ± 3.8 (5) |
| 1 | 17.9 ± 5.6 (3) | 21.7 ± 5.0 (4) |
| 2 | 22.5 ± 3.1 (3) | 26.2 ± 7.1 (3) |
| 3 | 24.7 ± 4.4 (3) | 34.6 ± 4.6 (3) |
| 4 | 24.8 ± 5.1 (3) | 33.1 ± 2.2 (3) |
| 12 | 21.2 ± 6.8 (5) | 31.2 ± 3.3 (3) |

*Results and discussion.* Determination of erythrocyte glutathione by the alloxan method, using blood from 10 dystrophic and 11 control animals, gave a value of 231 ± 40 μmoles per 100 ml in control animals and 348 ± 53 in dystrophic animals, constituting a 50% increase. Although our figures for erythrocyte glutathione content are lower than those reported by Ryerson *et al.*(3) they confirm the finding by these authors of a substantial increase in dystrophic rabbits.

Table I shows that average specific activity (s.a.) of erythrocyte glutathione isolated with carrier glutathione was higher in the experimental group at all time periods. This increase is not statistically significant at 0.5, 1 and 2 hours after injection of the labeled glycine, but is significant ($P<0.05$) at 3, 4

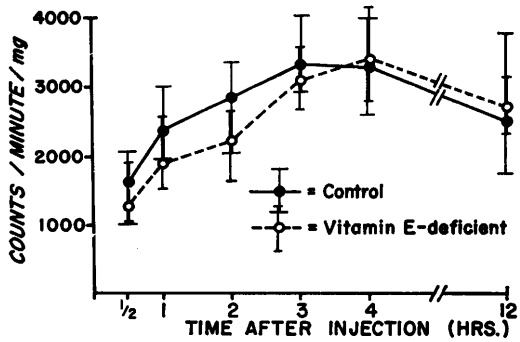


FIG. 1. Calculated specific activity of carrier-free erythrocyte glutathione. Curves are drawn through mean values. Vertical bars indicate range.

and 12 hours. As erythrocytes of the 2 groups contain different amounts of GSH, the s.a. of the mixture of erythrocyte GSH plus carrier GSH is not representative of the s.a. of the original erythrocyte GSH. Using the equation for inverse isotope dilution(20) it was possible to calculate the s.a. of the carrier-free GSH, as the amount of GSH per ml red blood cells, the amount of nonradioactive GSH added, and the s.a. of the isotopic mixture were known. In some instances the GSH concentration of glycine-$C^{14}$ injected animals was not determined. In such case the average GSH concentration in erythrocytes of the particular group was used to calculate the s.a. of the original erythrocyte GSH.

The result of these calculations is presented in Fig. 1. The trend of the data obtained for the experimental group is somewhat lower than control data during the first 3 hours after injection and somewhat higher at 4 and 12 hours, but differences between the 2 groups are not significant at any one time interval. If all other factors which can influence the s.a. of erythrocyte GSH after a glycine-$C^{14}$ injection were equal in the 2 groups, one should expect that the 50% higher concentration of GSH in the dystrophic animals would result in an approximately 50% lower s.a. of the carrier-free GSH. Fig. 1 shows that the s.a. of the erythrocyte GSH of the experimental group was much higher than that. Standard deviations are such that a 50% difference between the 2 groups could have been detected.

This higher than expected GSH activity could have more than one cause: (A) Rate of erythrocyte GSH synthesis could be higher in the dystrophic animals, (B) the s.a. of the glycine precursor could be higher in the dystrophic animals. Possibility (A) would give an explanation for the higher concentration of erythrocyte GSH found in dystrophic animals. To investigate possibility (B) it was necessary to determine the specific activity of the free glycine pool. Although the ability of red blood cells to synthesize GSH has at times been disputed, recent work has convincingly shown that mature red cells do synthesize GSH(6,7,21). Glycine for GSH synthesis in red blood cells is presumably taken up from the plasma. If the s.a. of unbound plasma glycine in dystrophic animals were higher than in control animals, this should result in a higher erythrocyte GSH activity. Table II shows the s.a. of unbound plasma glycine of some dystrophic and control animals, determined without use of carrier glycine. Although these results were obtained with a limited number of animals, it seems safe to conclude that the s.a. of plasma glycine was not higher in the dystrophic group.

The possibility of increased s.a. of plasma glycine has thus been excluded. However, it is conceivable that the s.a. of unbound glycine within the red cell is higher in the dystrophic animal. Differences in permeability of the red-cell membrane to glycine or a smaller glycine pool in the red cell could account for this. Since the specific activity of plasma glycine was not appreciably different in the 2 groups, a higher s.a. of unbound erythrocyte glycine should be reflected in a higher s.a. of whole blood glycine. Consequently, glycine was isolated from TCA-extracts of whole blood of 2 control and 2 dystrophic animals at 4 hours after injection of

TABLE II. Specific Activity of Unbound Plasma Glycine. Each figure is average of a duplicate determination.

| Time after inj of glycine-1-$C^{14}$, hr | Control rabbits, cpm/$\mu$g | Vitamin E-deficient rabbits, cpm/$\mu$g |
|---|---|---|
| .5 | 128 | 118 |
| 1 | 28, 70 | 21, 24 |
| 4 | 26 | 19 |
| 12 | 7 | 8 |

glycine-1-$C^{14}$. Glycine isolated from the 2 controls had an s.a. of 22 and 25 c.p.m./$\mu$g and that from the 2 dystrophic animals 19 and 23 c.p.m./$\mu$g. It may thus be concluded that the s.a. of the glycine precursor was not affected by the dystrophic condition which rules out possibility (B).

Calculation of the turnover rate of GSH would require extension of these experiments to longer time intervals after injection. Studying the turnover rate of rat erythrocyte GSH, Mortensen *et al.*(8) found a maximum incorporation of glycine-2-$C^{14}$ at one hour after injection and a half-life of 65 hours. Fig. 1 shows maximum incorporation for both dystrophic and control rabbits at 3-4 hours after injection. Variability of results was too great to permit half-life calculation with any degree of accuracy by extrapolation of the curve beyond the 12-hour interval. Unfortunately, young Vit. E-deficient rabbits do not survive the stage of acute muscular dystrophy very long, and time intervals of several days after injection of the labeled amino acid would involve the risk of losing most of the animals before completion of the experiment. In the absence of such turnover data, and on the basis of the results here presented it may be assumed that the higher erythrocyte GSH levels of dystrophic rabbits are caused by an increased rate of GSH synthesis. It has been suggested for both glutathione(22) and Vit. E(23) that they play a role in maintaining the structural integrity of the red cell. The observation that the rabbit responds to a lack of dietary Vit. E with increased erythrocyte GSH synthesis points to the existence of a compensatory mechanism. This recalls the finding by Prunty and Vass(24) that concentration of erythrocyte GSH varies inversely with that of plasma ascorbic acid. Prunty suggested that the functional diminution consequent on the lack of ascorbic acid could be counterbalanced by the GSH, explaining why scurvy takes a long 'latent period' to become manifest on a scorbutic diet. In a similar way GSH may counterbalance some of the deleterious effects of Vit. E-deficiency.

In view of the fact that animals in the control group did not gain as much weight as do rabbits on a commercial chow diet, our results must be interpreted with caution. Possibly, essential nutrients other than Vit. E were lacking in the semi-synthetic diet employed. Since the only nutritional difference between the 2 groups of animals was the supplementation of the control group with Vit. E it is not unreasonable to assume that the observed effect on glutathione metabolism was due to the lack of Vit. E in the experimental group. As Hove and Herndon(25) have pointed out, very little is known about the quantitative requirements of the rabbit for minerals and vitamins. Wooley and Mickelsen(26) have reported drastic effects of the variation of dietary fat, protein and mineral content on the weight gain of young rabbits. Additional work on glutathione metabolism is required in which the possible effects of other dietary variations in both Vit. E-deficient and Vit. E-supplemented animals are investigated.

*Summary.* 1. Compared with Vit. E-supplemented rabbits, average red blood cell GSH levels of dystrophic rabbits not receiving Vit. E supplements were found to be 50% higher. 2. In spite of this higher concentration of GSH in the dystrophic group the s.a. of GSH after injection of glycine-1-$C^{14}$ was not significantly different in the two groups, indicating a higher rate of glycine incorporation in the dystrophic group. 3. Specific activity of unbound glycine in plasma and whole blood was approximately the same in the two groups. This indicates that the higher incorporation of glycine-1-$C^{14}$ into erythrocyte GSH of dystrophic animals was not caused by a higher s.a. of the glycine precursor pool.

1. Tallan, H. H., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 553.

2. Azzone, G. F., Carafoli, E., Barbolini, G., *Atti Acad. Naz. dei Lincei,* 1957, v22, 763. *Musc. Dyst. Abstr.,* 1958, v2, 337.

3. Ryerson, S. J., McMillan, P. J., Mortensen, R. A., *J. Biol. Chem.,* 1958, v233, 1172.

4. Gyorgy, P., Rose, C. S., *Science,* 1948, v108, 716. Rose, C. S., Gyorgy, P., *Am. J. Physiol.,* 1952, v168, 414.

5. Tsen, C. C., Collier, H. B., *Can. J. Biochem. Physiol.,* 1960, v38, 981.

6. Dimant, E., Landsberg, E., London, I. M., *J. Biol. Chem.*, 1955, v213, 769.

7. Elder, H. A., Mortensen, R. A., *ibid.*, 1956, v218, 261.

8. Mortensen, R. A., Haley, M. I., Elder, H. A., *ibid.*, 1956, v218, 269.

9. Young, J. M., Dinning, J. S., *ibid.*, 1951, v193, 743.

10. Diehl, J. F., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 657.

11. ———, *Arch. Biochem. Biophys.*, 1962, v99, 148.

12. Diehl, J. F., Kistler, B. G., *J. Nutrition*, 1961, v74, 495.

13. Diehl, J. F., Sanders, L. L., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 8.

14. Waelsch, H., Rittenberg, D., *J. Biol. Chem.*, 1941, v139, 761.

15. Patterson, J. W., Lazarow, A., in *Methods of Biochemical Analysis*, D. Glick, edit., Interscience Publishers, New York, 1955, v2, 273.

16. Gabbe, E., *Klin. Wochenschr.*, 1929, v8, 2077.

17. Diehl, J. F., *Anal. Chem.*, 1959, v31, 1204.

18. Block, R. J., Weiss, K. W., *Amino Acid Handbook*, Chas. C Thomas, Publ., Springfield, Ill., 1956, p94.

19. Rosen, H., *Arch. Biochem. Biophys.*, 1957, v67, 10.

20. Overman, R. T., Clark, H. M., *Radioisotope Techniques*, McGraw-Hill, New York, 1960, p396.

21. Kasbekar, D. K., Sreenivasan, A., *Biochem. J.*, 1959, v72, 389.

22. Fegler, G., *Nature*, 1952, v170, 624.

23. Collier, H. B., McRae, S. C., *Fed. Proc.*, 1955, v14, 195.

24. Prunty, F. T. G., Vass, C. C. N., *Biochem. J.*, 1943, v37, 506.

25. Hove, E. L., Herndon, J. F., *J. Nutrition*, 1957, v63, 193.

26. Wooley, J. G., Mickelsen, O., *ibid.*, 1954, v52, 591.

Received August 1, 1962. P.S.E.B.M., 1963, v112.

## Induction of Prolonged Latency in Psittacosis Infected Cells by Aminopterin.* (27947)

MORRIS POLLARD AND NEHAMA SHARON

*Lobund Laboratory, University of Notre Dame, Notre Dame, Ind.*

The phenomenon of virus latency was surveyed thoroughly in 1957(1). Viruses were not detectable when inoculated into newborn mice (lymphocytic choriomeningitis), or into clones of cells which survived the cytolysis of earlier viral infection (Newcastle disease virus), or into lines of cells which were visibly unaltered while producing virus (mumps). Mature psittacosis virus was not detected in cultures of nutritionally depleted "L" cells; however, infectious virus emerged from such cultures after treatment with amino acids and water soluble vitamins(2). Viral elements survived in such nutrient-depleted cultures for 8 days.

Psittacosis virus has been studied extensively by acridine orange fluorochrome staining of infected tissue cultures(3,4). This technic provided a visible cytochemical record of viral activity, as influenced by antimetabolites which modified the normal sequence of biosynthetic reactions associated with replication. Each virus particle maturated through a clearly defined sequence of cytochemical changes which reflected nucleic acid metabolism of the virus. The infective DNA virus particle entered the cytoplasm, became surrounded by a matrix of RNA, and from the resulting inclusion increased numbers of infectious DNA-staining virus particles emerged. The changes observed during this predictable virus maturation sequence reflected accurately the infectious status of the virus. During the period in which the virus inclusion was observed as a mass of RNA-staining material ("red ball"), it was not infectious and it represented an eclipse or latent stage of the virus.

Addition of antimetabolite drugs to the culture medium of infected cells interrupted the replication sequence of the virus; this effect was related to the stage of replication at

* Supported by grant from U. S. Public Health Service.