Effect of Diet and Trace Minerals on Erythrocyte Fragility and x-Irradiation Mortality in Rats.* (20991)

MARGUERITE A. CONSTANT AND PAUL H. PHILLIPS.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Several studies have shown that hemorrhage or loss of red blood cells into the lymphatics frequently were responsible for the severe anemia seen post-irradiation(1). Evidence of increased red cell destruction has also been obtained(2). Chanutin *et al.*(3) observed that whole body irradiation (500 r) caused an increased red cell destruction following phenylhydrazine treatment. Erythrocyte fragility determinations were not made. Goldschmidt *et al.*(4) found that the fragility of rat erythrocytes reached a maximum 12 hours post-irradiation (600 r) but had returned to normal in 24 to 48 hours. Recent studies by Harman(5) have shown that the erythrocytes of rats fed stock diet were more fragile than the erythrocytes of rats fed a semi-purified diet. It appeared desirable, therefore, to study the effect of diet, starvation or whole body irradiation on erythrocyte fragility in order to correlate, if possible, the ease of destruction of erythrocytes and the lethality of whole body irradiation.

Experimental. Weanling male rats of the Sprague-Dawley strain received food and water *ad libitum* and were given 2 drops of haliver oil weekly. Three basal diets were used in these experiments. Diet 1 was Rockland guinea pig diet which contained 17% protein and 2.5% fat (minimum values). Diet 2 was composed of 45% corn flakes, 20% wheat flakes, 12% soybean oil meal, 12% meat scraps, 5% alfalfa, 3% brewer's yeast (dried), 3% fish solubles and 0.01% irradiated yeast (22% protein, 3% fat). Diet 3 was a semi-synthetic diet composed of 18% crude casein, 4% Salts IV, 68% sucrose, 10% corn oil or Crisco and B vitamins. The B vitamin supplement contributed, in mg/kg of ration, thiamine HCl, 3.8; pyridoxine HCl, 3.8; riboflavin, 4.5; niacin, 38.0; Ca-pantothenate, 30.0; inositol, 100; biotin, 0.1; folic acid, 0.2; and 1.0 g choline. The trace mineral supplements contained 1.0 to 0.5 mg Co, 0 to 2.0 mg Cu and 0 to 7.5 mg Mn per 10 g of ration depending upon the experiment as indicated. Samples of blood were taken from the heart under nembutal anesthesia (6 mg/100 g of body weight, intraperitoneally). Erythrocyte fragilities were determined by the method of Harman(5). Determinations on blood samples from an animal in the control and each experimental group were made simultaneously. The concentration of the hypotonic solutions were graded stepwise by 0.03% from 0.35% (Exp. 1) or 0.02% from 0.36% (Exp. 2 and 3) to 0.50%. Since all solutions in these ranges gave similar results data are reported only for representative concentrations. The amount of ***whole body irradiation*** administered to animals in each experiment was 700 r delivered from a 200 KV Westinghouse therapeutic machine at the rate of 20 r/minute, 175 KV, 10 ma, 1 mm Al, 0.2 mm Cu, HVL 0.83 mm Cu, 40 cm from target.

Results. The first series of experiments was a study of the effect of starvation and trace minerals on erythrocyte fragility. Diets 1 (natural), 3 (synthetic) and 3 plus 10 mg % Co, 2 mg % Cu, and 5 mg % Mn were used. Although fragility determinations were not reported by Harman(5) until after 3 months of feeding, it was apparent from our data that the difference in osmotic fragility of erythrocytes due to diet was evident at 35 days. By the 41st day (Table I) the rbc's of the majority of animals fed diet 3 were 50% less fragile. Starvation periods of 8 to 24 hours did not affect the degree of fragility. The hemoglobin values at this time were 13.8 ± 0.8, 13.4 ± 0.3, and 17.3 ± 0.8 g for animals fed diets 1, 3 and 3 plus trace minerals respectively. Fragility data obtained 24 and 120 hours post irradiation showed that the greater osmotic stability of rbc's of rats

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TABLE I. Effect of Diet and Starvation on Erythrocyte Fragility and Mortality from X-Irradiation.

	% hemolysis in 0.41% NaCl			% hemolysis in alloxan§		Survivors
Day	41*	46†	50‡	46	50	+20
Hr starved	0–24	24	120	24	120	—
Diet No.						
1 (natural)	74 ± 7	97 ± 5‖	82 ± 11	51 ± 7	57 ± 4	
1 + irradiation		93 ± 5	77 ± 20	54 ± 7	54 ± 11	1
3 (synthetic)	35 ± 12	60 ± 11	45 ± 9	16 ± 7	29 ± 5	
3 + irradiation		62 ± 12	28 ± 9	13 ± 9	27 ± 6	3
3 + Co, Cu, Mn	29 ± 7	53 ± 6	62 ± 16	9 ± 4	27 ± 6	
3 + *idem* and irradiation		49 ± 11	50 ± 22	10 ± 8	21 ± 4	3

* 12 rats/series.
† 24 hr post-irradiation; 4 rats/control, 8/irradiated series.
‡ 4 rats each series, 4 for survival.
§ Increased hemolysis in 25 min. in alloxan (10 mg % in .50% NaCl) compared to 30 min. in .50% NaCl.

$$\| \pm \sqrt{\frac{\Sigma x^2}{N}}.$$

fed diets 3 and 3 plus Co, Cu and Mn was still evident. Prolonged starvation (120 hours) did not alter the fragility. The rbc's of irradiated animals were not more fragile than the rbc's of control animals. An explanation for the general increase in rbc fragility seen in all groups cannot be given at this time. Temperature and pH among other factors have been shown by Parpart *et al.*(6) to affect fragility. Venous rbc's were observed to be more fragile than arterial rbc's from the same animal but that difference was not sufficient in magnitude to invalidate the differences caused by dietary treatment. The poor survival rate of animals fed diet 1 indicated that animals with more fragile rbc's may be more susceptible to irradiation effects.

A second series of experiments was made to determine whether an increased fragility of rbc's and poor survival rate of animals fed a different natural diet would occur in comparison to results with the synthetic diet. The effect of diets 2 (natural) and 2 plus trace minerals was compared with that of diets 3 and 3 plus trace minerals. The level of Co, Cu and Mn used initially in this experiment was 10 mg %, 4 mg % and 15 mg % respectively. Since it was observed that the

TABLE II. Effect of Diet, Dietary Trace Minerals and Irradiation on Erythrocyte Fragility and Mortality.

	% hemolysis in 0.40% NaCl						Survivors	
Day	18*	29*	39	45	54	60†		
Hr starved	0	0	24–48	24–48	0	24	+15	+30
Diet No.								
2 (natural)	51 ± 13	35 ± 24	69 ± 11	56 ± 17	62 ± 13	78 ± 8	6	4
2 + Co, Cu, Mn	44 ± 16	31 ± 14	45 ± 9	33 ± 9	30 ± 8	48 ± 10	1	1
3 (synthetic)	31 ± 12	9 ± 4	33 ± 9	19 ± 9	18 ± 6	28 ± 9	7	5
3 + *idem*	34 ± 11	16 ± 8	44 ± 10	32 ± 10	35 ± 9	41 ± 9	5	4
	Increased hemolysis in alloxan solution							
2	39 ± 6	31 ± 8	25 ± 5	38 ± 12	28 ± 8	39 ± 13		
2 + *idem*	35 ± 8	28 ± 9	17 ± 5	19 ± 6	9 ± 3	18 ± 5		
3	21 ± 6	19 ± 10	7 ± 2	12 ± 5	6 ± 2	12 ± 6		
3 + *idem*	23 ± 3	13 ± 6	10 ± 3	15 ± 4	10 ± 3	14 ± 3		

* 6 rats/series (0.41% NaCl) day 18–29, 8 rats/series day 39 to +30.
† 24 hr post-irradiation.

TABLE III. Effect of Dietary Cobalt on Erythrocyte Fragility and Mortality in the Rat. 10 rats/series.

Diet No.	Concentration of solutions .36% % hemolysis	.38% % hemolysis	.40% % hemolysis	Alloxan, % hemolysis*	No. surviving irrad.† Days 15	Days 30
Exp. 3A—0 hr starvation; day 26						
1	94 ± 5	86 ± 6	70 ± 12	42 ± 8		
1 + Co	88 ± 6	83 ± 8	73 ± 13	46 ± 6		
2	70 ± 8	47 ± 13	25 ± 11	19 ± 7		
2 + Co	48 ± 16	36 ± 12	23 ± 9	13 ± 7		
3	60 ± 16	42 ± 15	21 ± 12	15 ± 8		
3 + Co	77 ± 11	62 ± 16	43 ± 16	26 ± 10		
Exp. 3B—0 hr starvation; day 46						
1	90 ± 6	77 ± 10	56 ± 14	35 ± 8	4	3
1 + Co	70 ± 11	58 ± 12	43 ± 11	22 ± 7	3	3
2	88 ± 7	78 ± 8	56 ± 13	28 ± 8	7	7
2 + Co	39 ± 10	27 ± 8	18 ± 6	6 ± 2	5	3
3	71 ± 6	52 ± 7	28 ± 6	12 ± 5	8	5
3 + Co	64 ± 12	51 ± 13	31 ± 9	13 ± 4	1	1

* Increased hemolysis in 25 min. in alloxan (10 mg % in 0.50% NaCl) compared to 30 min. in 0.50% NaCl.

† Separate groups of 8 animals each were used for mortality study.

food became rancid after a 48 hour exposure to air, the level of trace minerals was halved and the corn oil was replaced by Crisco at the end of the first week. The cobalt level only was increased to 10 mg % on the 33rd day. No rancidity was detected. A decreased fragility of rbc's of rats fed diet 3 was again observed and was measurable on the 29th day (Table II). Starvation periods up to 48 hours were again found to have no effect. The data obtained on the 39th day showed that the fragility of rbc's of rats fed diet 2 (natural) plus Co, Cu and Mn had decreased and was comparable to that of rbc's of animals fed the synthetic diets, a fact still evident 24 hours post-irradiation. The decreased fragility of rbc's of animals fed the supplemented diet 2, diet 3 and the supplemented diet 3 compared to that of animals fed diet 2 only was also evident in the alloxan solutions. The mortality data in Table II showed that 700 r was greater than an LD_{50} dose only for animals fed the supplemented diet 2.

A third experiment was made to determine the effect of dietary cobalt upon erythrocyte fragility and mortality. Cobalt was fed at a level of 10 mg %. The diets fed were 1) diet 1, 2) diet 1 + Co, 3) diet 2, 4) diet 2 + Co, 5) diet 3, and 6) diet 3 + Co. The level of fat in the synthetic diets (lots 5 and 6) was reduced to 5% in order to have this dietary constituent comparable in all diets. Hemolysis data (Table III) obtained on the 26th day showed that the erythrocyte fragility of animals fed diet 1 was greater than that of animals fed diets 2 or 3. No effect of cobalt on erythrocyte fragility was seen in any of the groups. By the 46th day the fragility of rbc's of rats fed diet 2 had increased and was comparable to that of animals fed diet 1. A marked reduction in the fragility of rbc's of animals fed the cobalt supplemented diet 2 was observed. The supplementation of diet 1 with Co had no effect on erythrocyte fragility by the 46th day. Although the fragility of rbc's of rats fed diet 3 with 5% fat was somewhat higher than observed previously, Co supplementation was without effect on erythrocyte fragility.

The mortality data in Table III showed that 700 r was again more than an LD_{50} dose for animals fed diet 1, diet 1 plus Co, or diet 2 plus Co and was less than an LD_{50} dose for animals fed diet 2 only or diet 3 only. The rapid mortality rate of animals fed the supplemented synthetic diet was unexpected in view of the findings of the first two experiments. Since the only difference in the syn-

thetic diets used in this and the previous experiments was the dietary level of fat, the data indicate that the decreased level of dietary fat in the cobalt supplemented synthetic diet might have been responsible for the increased mortality rate.

Discussion. The observation reported by Harman(5) that rbc's of rats fed a natural diet were more fragile than the rbc's of rats fed a synthetic diet was confirmed and extended by these studies.

In addition it was found that the supplementation of diet 2 (natural) with Co, Cu and Mn or cobalt only caused a marked reduction in erythrocyte fragility. A reduction in fragility of rbc's from animals fed the cobalt supplemented diets 1 (natural) or 3 (synthetic) was not observed. These data showed that the effect of trace minerals in reducing the erythrocyte fragility in the tests referred to earlier in this report was due to the dietary cobalt. These data also showed that the action of cobalt in reducing erythrocyte fragility was pronounced only and associated with the feeding of diet 2.

Because of the effect of cobalt on cellular respiration and the production of polycythemia, Parr *et al.*(7) fed a cobalt supplemented diet to mice for 8 days prior and 15 days following irradiation. This supplement, which was prepared by immersing pellets in a 2% $CoCl_2$ solution, afforded protection to the treated animals. Our studies with rats indicate that prolonged treatment of animals before and after irradiation with cobalt may be detrimental.

A summary analysis of the mortality data obtained in these experiments showed that 700 r was an LD_{67} for animals fed diet 1 (7/12 dead on 15th day); an LD_{63} for animals fed the supplemented diet 1 (5/8 dead on 15th day); an LD_{75} for animals fed the supplemented diet 2 (10/16 dead on 15th day); and $LD_{31\text{-}38}$ for animals fed diets 2 only or 3 only. These data indicate that the pre-irradiation fragility of erythrocytes was of less importance in determining the lethality of irradiation than the type and adequacy of the diet.

In the second series of experiments the incisors of all animals fed the supplemented diet 2 were observed to be almost completely depigmented in approximately 2 to 3 weeks. The incisors of all other animals were normal. A similar observation was made in the third experiment and included several of the animals fed the supplemented diet 3. The incisors of animals fed diet 1, diet 1 plus Co, diet 2 and diet 3 were normal. Additional precautions were made to assure adequate vitamin A intake by doubling the haliver oil supplement. This procedure was without effect. Rose and Gyorgy(8) have reported an increased fragility of rbc's of rats mildly deficient in vit. E. However, a decreased fragility of rbc's of animals fed the supplemented diet 2 was observed. These data indicate that the bleaching effect caused by the supplement was probably not due to avitaminosis A or avitaminosis E. An explanation for this bleaching effect cannot be given at this time.

Summary. The data indicate that the fragility of erythrocytes pre-irradiation was of less importance in determining the lethality of irradiation than the type and adequacy of the diet. The three basal diets used in these studies supported growth equally well; however, animals fed diet 1 (natural) succumbed to the effects of irradiation more rapidly than did animals fed diet 2 (natural) (rbc's equally fragile) or those fed diet 3 (synthetic) (rbc's less fragile). Dietary trace mineral supplementation was observed to reduce the erythrocyte fragility. This effect which was traced to the cobalt of the supplement was pronounced and was associated with the feeding of one of the natural diets (diet 2). Although the rbc's of animals fed the supplemented diet 2 were less fragile, these animals showed an earlier and more rapid mortality rate than did animals fed the basal diet. The data from the present studies indicate that long term feeding of polycythemic levels of cobalt increased the mortality rate of animals given whole body irradiation.

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Prolongation of the Lag Phase of Irradiated *Escherichia coli*. (20992)

E. BEUTLER, MELBA J. ROBSON, AND L. O. JACOBSON.

From the Department of Medicine and Argonne Cancer Research Hospital, The University of Chicago, Chicago, Ill.

Although the lethal effect of ionizing radiations on bacteria has been the subject of repeated and extensive study(1), the recovery of damaged organisms has received relatively little attention. In the course of attempts to modify the lethal effect of X rays on bacteria, we have observed a delay in the onset of multiplication of the *surviving* organisms. Because recovery from radiation injury in higher animals may well be dependent on the resumption of growth by damaged cells rather than on the revival of "killed" cells, we believed that this aspect of the problem deserved further investigation.

Materials and methods. Bacteria. A culture of *Escherichia coli*,* serially passed on blood agar plates and stored in a refrigerator, was used throughout these studies. Unless otherwise stated, all cultures were incubated aerobically at 37°C. Pour plates were made by adding one cc of culture, diluted to produce approximately 50 to 200 colonies, to 8 cc of melted tryptocase soy agar† at 40 to 44°C. Colony counts were made after an 18-hour incubation period. Reincubation of some plates from both irradiated and non-irradiated cultures for additional 24- and 48-hour periods failed to give any increase in colony counts. *Irradiation.* Cultures were irradiated in Evelyn spectrophotometer tubes with 250-kvp X rays at 15 ma with .25-mm Al filtration. The tubes were held in an ice water bath by a modification of the apparatus described by Stapleton *et al.*(2). Exposure rates, calibrated with a Victoreen thimble chamber inside of an Evelyn tube, varied from 425 to 480 r per minute. In each experiment, a single overnight culture of *E. coli* in tryptocase soy broth served as the source of both control and irradiated bacteria. Except where otherwise indicated, the original culture was diluted sufficiently in tryptocase soy broth prior to irradiation so that pour plates could be made from irradiated and control cultures without further dilution. Because the survival ratio of the strain of *E. coli* employed was approximately one in 2500 at 30,000 r, the non-irradiated cultures were diluted 10^3- and 10^4-fold further than the cultures receiving this exposure. All cultures were kept in ice water baths from the time of dilution, throughout irradiation, and until after the initial pour plates had been made after irradiation. After pour plates had been prepared from irradiated and control cultures, they were placed into a 42°C water bath and warmed for 5 minutes prior to transfer to a 37°C incubator. The control and irradiated cultures were handled identically in all respects throughout: equal volumes were moved in and out of the water baths and incubator in equal-sized tubes.

Results. The growth of irradiated and non-irradiated control cultures was studied repeatedly. Typical growth curves are shown in Fig. 1. Cultures that had been X-irradiated with 30000 r consistently showed a ¾- to 1½-hour delay in the onset of the phase of logarithmic

* Isolated from a patient and supplied to us through the kindness of Miss Dorcas Fasan, Department of Pediatrics, University of Chicago.

† Baltimore Biological Laboratory, Baltimore, Md.