acid, but direct evidence is not available from our experiments.

The study of the effects of CO_2 on bacterial metabolism entails certain difficulties. In testing the essentiality of CO_2 for growth, it is obviously insufficient merely to omit it from the medium, and considerable care must be exercised to remove it. The possibility always exists that traces of CO_2 have not been removed; therefore, any experiments which show a positive growth response in the supposed absence of this ubiquitous compound must be re-checked several times in order to insure that the CO_2 has really been removed. In fact, it is risky to claim the existence of specific nutritional requirements in the complete absence of CO_2, since such a condition can never be proved unequivocally, and all that the investigator can do is to reduce the level of adventitious CO_2 to a minimum.

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Effect of Gamma Irradiation on Response of Fasted Rats to Erythropoietin.* (30387)

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We have reported(1) that the fractional reduction of iron uptake by erythropoietic tissue, produced by irradiation, was much less in rats exposed when their erythropoiesis was maximally stimulated than when it was normal(1). In this same report it was postulated that the high endogenous levels of erythropoietin (EP) observed(2) in the stimulated rats might explain in part the results obtained. Furthermore, preliminary results(1) showed that the response to erythropoietin was much greater when EP was given 5 hours before irradiation than when it was given five hours after. The object of

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TABLE I. ^{59}Fe Distribution in RBC, Femur and Plasma 3 Hours After Tracer Injection in: Normal Rats, Rats Fasted for 4 Days and Rats Fasted for 4 Days and Injected After 2 Days of Fasting with 20 U Erythropoietin (EP).

Group	^{59}Fe, % dose			PITR, %/hr
	Plasma	RBC	Femur	
Normal (12)	15.40 ± .64	4.60 ± .34	6.0 ± .13	60 ± 4.7
Fasted (20)	57.10 ± .88	.35 ± .03	1.66 ± .08	18.5 ± .5
Fasted + 20 U EP (20)	2.84 ± .24	13.30 ± .88	8.20 ± .08	118.0 ± 2.75

± = standard error.

PITR = plasma iron turnover rate: $\frac{1}{3} \ln \frac{1}{^{59}Fe_{Pl_3}}$.

$^{59}Fe_{Pl_3}$ = fraction injected tracer remaining in plasma at 3 hr.

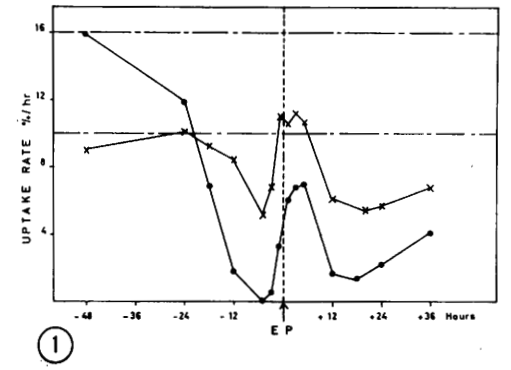
No. of animals per group indicated within parentheses.

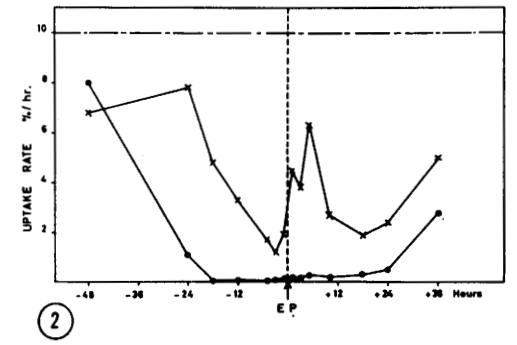
this communication is to describe results of experiments designed to study the radio sensitivity of the response to doses of erythropoietin of 5 and 20 U given before and after irradiation, and to explore the effects of irradiation on response to erythropoietin as a function of the interval between irradiation and erythropoietin injection.

Material and methods. Male A × C rats obtained from the Instituto de Biología Juan Noé, weights ranging between 160-175 g, were used. The animals were fasted for a total of 4 days. Erythropoietin was given after 2 days of fasting and the tracer dose of ^{59}Fe used to study ^{59}Fe distribution(3) was injected at the end of the period of fasting. Gamma irradiation ^{137}Cs, dose rate 6 r/min(4), was given at different intervals before or after EP injection. EP injections were given subcutaneously, 5 rats were used per group. A large pool of plasma of phenylhydrazine treated rabbits (Hb = 2.6 g/100 ml) 10 U Standard B erythropoietin/ml was used as a source of EP. The activity of the plasma was bioassayed for its effect on RBC ^{59}Fe uptake(5) against erythropoietin St. B., (kindly supplied by Dr. Bangham, Division of Biological Standards, National Institute for Medical Research, London) using a 4-point design(6). The plasma was kept frozen in 10 ml vials until used. As Keighley has shown(7) and we have confirmed, activity keeps well under these conditions for over a year.

The response of fasted rats to erythropoietin was determined using the 3-hour ^{59}Fe distribution test(3), which measures ^{59}Fe in plasma, circulating erythrocytes (RBC) and femurs, 3 hours after tracer injection. From these data estimates can be made of the iron uptake rate by femurs (marrow) and RBC.

Results. A. Radiosensitivity of response to EP in relation to interval between EP and irradiation. Fasting for 4 days markedly reduces ^{59}Fe uptake by femurs and RBC, and reduces the plasma disappearance rate. EP injection produces a marked increase in uptake by RBC and femurs and accelerates plasma disappearance rate (Table I). These observations are in agreement with our previously published findings(3). Irradiation with 100-200 r as we have shown previously, does not further change the iron distribution pattern in fasted rats(1) which show residual iron uptakes in RBC and femurs similar to those seen in 600-700-800 r irradiated normal rats. The effects of irradiation on the response to EP depends on the moment irradiation is given in relation to EP. This can be seen in Figs. 1 and 2 which show plots of the iron uptake rate by femurs and RBC of irradiated (100 and 200 r) EP treated rats, in relation to time of EP injection. There are clearly 2 periods when maximum effects of irradiation are obtained, one around 3-5 hours before EP injection and the other 10-24 hours after EP injection. The effects of radiation are least when given 0-5 hours after EP injection and 24-48 hours prior to injection.





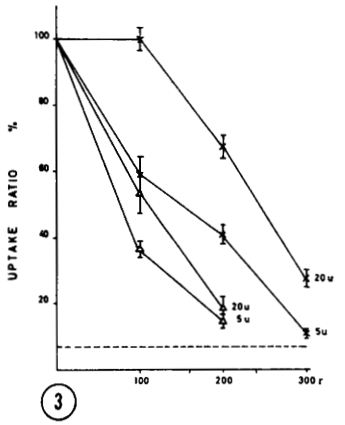


FIG. 1. Ordinate: Iron uptake rate by femurs ×——× and RBC ●——●. Abscissa: Time of irradiation (100 r) in relation to EP injection. Iron uptake rate calculated as:

$$\frac{\text{PITR} \times \%\ {}^{59}\text{Fe(Femur or RBC)}_3}{1 - {}^{59}\text{Fe}_{\text{Pl}_3}}$$

Where: PITR = plasma iron turnover rate, see Table I.

${}^{59}\text{Fe}_{\text{Pl}_3}$ = fraction of injected tracer remaining in plasma at 3 hours.

FIG. 2. Ordinate: Iron uptake rate, femurs ×——× and RBC ●——●. Abscissa: Time of irradiation (200 r) in relation to EP injection. Iron uptake rate calculated as in Fig. 1.

B. Radiosensitivity of the response at 2 dose levels of erythropoietin.

Fig. 3 shows a plot of the ratio of iron uptake rate of femurs of irradiated to that of non-irradiated EP treated rats, as a function of radiation dose up to 300 r. In the animals irradiated 5 hours before erythropoietin injection the femoral uptake ratio drops off to very low values at 300 r and there is no marked difference in the curves obtained with 5 or 20 U of erythropoietin. On the other hand, when erythropoietin is given 5 hours before irradiation, the depression produced by irradiation is much less the higher the EP dose and a clear shoulder appears on the curve for 20 U.

Discussion. The results described clearly indicate that irradiation affects the response to EP of erythropoietic tissue differently depending on the time interval separating the two and on whether radiation is given before or after EP. The response is least affected when EP is given 5 hours before irradiation and most affected when it is given 3-5 hours after. When EP is given 5 hours before irradiation, the effect of irradiation is smaller the greater the dose of EP. There is no marked influence of EP dose when irradiation is given 5 hours before EP. These results obtained with anemic rabbit plasma containing high concentrations of EP, agree with our previous observations(1) in which Armour Erythropoietin was used, and suggest that the observed changes can be ascribed to erythropoietin. In the following paragraphs we will discuss the effects of irradiation on the response to EP given after irradiation, and the effects when irradiation is given prior to EP.

FIG. 3. Ordinate: Ratio of femoral iron uptake rate of irradiated EP treated to that of non-irradiated EP treated rats. Abscissa: Dose in roentgens.

×——× Mean ± 1 SE for rats irradiated 5 hr after EP.

△——△ Mean + 1 SE for rats irradiated 5 hr before EP.

— — — Ratio for fasted rats and fasted rats irradiated 300 r 5 hr before EP.

Effects of irradiation given prior to EP. The effects of irradiation on the response to exogenous EP given after irradiation have been studied by Gurney and Lajtha(8), in transfused mice and by Porteous *et al*(9) to endogenous EP in fasted re-fed mice. In the analysis of these authors the response to EP is assumed to depend on the number of viable stem cells remaining after irradiation. Viewed in this manner the decrease of response to EP with time after irradiation could be interpreted as a fall off in the stem cell number, that is, as stem cells dying off with time after irradiation. If this interpretation is valid, the results obtained with 100 r (Fig. 1) suggest that if a stem cell affected by irradiation (which will eventually die) can interact with EP before it dies, it can differentiate into erythroid elements.

The early recovery of the capacity to respond to EP after irradiation (24-48 hours) observed in our experiments, could be a measure of the recovery of the viable stem cell pool, but more likely reflects a recovery of total stem cell number including cells incapable of sustained proliferation(10,11).

Effect of irradiation given after EP. The capacity of irradiation to depress the response to EP is minimum when given between 0-5 hours after EP. The data can be tentatively interpreted in at least two ways: a) "Interpretation" 1. Increased levels of erythropoietin protect non-specifically against radiation damage, at least as measured by the capacity to respond to EP. The fact that there is an EP dose effect (Fig. 3) could be considered as support for this view; there is, however, the discrepancy that 100 r given 1 hour before EP produces as little damage as 100 r given after EP when high EP levels occur. The 200 r data, are also not in too good agreement for effects of irradiation decrease with time up to 5 hours after EP when presumably concentration of EP is decreasing. b) "Interpretation" 2. Other systems which show marked changes in radiosensitivity in relation to interval between irradiation and the triggering event, are antibody synthesis(12) and liver regeneration(13,14). It is conceivable that in these cases, as in the case of the response to EP, irradiation interferes with nuclear RNA synthesis, and that once this process is under way radiosensitivity is decreased. There is evidence suggesting that EP produces increase of RNA synthesis *in vitro*(15,16) and *in vivo*(16).

Another factor which could conceivably play a part in the effects of high doses of EP in diminishing sensitivity to irradiation could be the action of EP in causing erythroid cells to follow the skipped division pathway(17), for a reduction in the number of divisions would conceivably reduce the probability of cell death as a consequence of irradiation.

The greater effectiveness of irradiation given 10-24 hours after EP presumably could reflect the radiosensitivity of the erythroid elements present, as a consequence of EP action, at the moment of irradiation.

Summary. The effects of γ-irradiation on the response to erythropoietin (EP) of fasted rats depend on the moment irradiation is given in relation to EP injection. Maximum depression of the erythropoietic response is obtained when irradiation is given 3-5 hours before and 10-24 hours after EP. Minimum effects are obtained when irradiation is given between 1-5 hours after and 24-48 hours before EP. When irradiation is given after EP there is a clear EP dose effect; this is not the case when irradiation is given prior to EP.

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Influence of Diet on Chronic Oral Toxicity of Safrole and Butter Yellow in Rats.* (30388)

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Chronic oral toxicity studies are generally conducted in dogs and rats according to methods described by Fitzhugh(1). Such methodology, while noting that dietary effects on the toxicity of chemicals represent a problem, does not prescribe the use of specific standardized diets. "Proved commercial diets" are usually employed and the composition of such diets varies considerably.

In the case of a relatively weak hepatic carcinogen such as Safrole(2,3,4,5), dietary composition may have significant bearing on the outcome of experiments, and a study was therefore made to determine hepatotoxic effects of Safrole as well as those of a more potent hepatic carcinogen, butter yellow, in rats placed on diets containing 5, 10 and 30% protein. Fat content was varied to 5 and 15% in the case of the 30% diet. One group of rats was also fed a riboflavin-free 10% protein, 5% fat diet to compare the butter yellow-carcinogenesis-enhancing effect of this deficiency with its effect on Safrole carcinogenesis.

Material, methods and experimental design. The animals used were Osborne-Mendel male rats obtained from Camm Research Institute. Animals received Achromycin†† (0.001%) in drinking water for three days on arrival. Starting weight averaged 114 g (range, 92-135 g) for each group of 10 rats. Food consumption measurements were made during the first year of the experiment. The animals were given 0.5% Safrole† or 0.06% butter yellow mixed in their diet. The evaporation rate of Safrole was found by chemical analysis to be 11.3% per 3-day period(3). The diets were prepared in 2-kg lots as needed, kept refrigerated in sealed jars, and fed freshly every third day. All animals were kept individually in hanging cages. Animal room temperature was 24°C (± 2°C), and tap water was given *ad libitum*. All rats were killed when they appeared to be moribund.

Autopsies were conducted on all animals and included measurement of organ weights and histological study of lungs and livers and of any organ which appeared upon inspection to be diseased. The experimental design and diets are shown in Table I.

Results. The results are illustrated in Fig. 1 to 5. Significant differences, where noted, indicate a probability level of 5% or less by Student's t-test.

Weight curves (Fig. 1) reveal that during the first year, the controls showed the anticipated growth variations dependent on the protein and fat content of the diet with only

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†† Achromycin was generously supplied by Lederle Laboratories Division of American Cyanamid Company, Pearl River, New York.

† Safrole was obtained from Matheson, Coleman & Bell, Rutherford, N. J., and p-dimethylaminoazobenzene came from K & K Laboratories, Inc., Plainview, N. J.