An equally informative analysis of the replication of DNA in vitro is also provided by the existence of this material.

Note added in proof: After this manuscript was submitted for publication, two papers appeared, providing evidence for the circularity of RF-DNA of ϕX147 (*17*).

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Erythropoietin Production Following Gamma Irradiation and Hemorrhage in Dogs

Abstract. *The production of erythropoietin in dogs increased after they had been exposed to 300 rad of gamma rays and then subjected to hemorrhage.*

The recovery of erythropoiesis which takes place in irradiated rats and dogs, and the possible role of erythropoietin as a stimulating factor in these irradiated animals has been discussed (*1*). In particular, it has been noted that bleeding has a favorable effect in irradiated animals (*2*). We thought it possible that one of the factors contributing to this favorable effect might be the increased titers of erythropoietin following bleeding. We therefore conducted experiments to test this hypothesis.

Nine mongrel dogs, each weighing approximately 10 kg, were divided into three groups. Group I was irradiated and immediately thereafter bled until 30 percent of the total blood volume was removed; group II was irradiated but not bled; group III was bled without prior exposure to radiation. All irradiated dogs received 300 rad of a 2 kc Co^{60} source over the whole body. During irradiation the animals were placed in specially designed cages providing a constant geometry and sufficient oxygen supply. The dogs were bled by cardiac puncture; blood for assay was taken from veins in the hind leg. Serum was separated by centrifuging at about 1200 *g* for 30 minutes.

For comparison, one female dog which had not been irradiated was made anemic by injections with a 2.5 percent aqueous solution of phenylhydrazine hydrochloride, a total of 35 ml being given over a 7-day period. The initial hemoglobin level of 20.2 g/100 ml of blood decreased to 9.8 g/100 ml. Serum was prepared as for the other dogs. Erythropoietic activity of plasma was assayed by a modified version of the method described by Plzak *et al.* (*3*).

Female Wistar albino rats (numbering 180) were divided into groups of five. Each rat was ready for use when it weighed approximately 160 g; all were starved for 24 hours before use, and until they were killed. Three intraperitoneal injections of 2 ml of dog serum were given in the course of 3 days. Six hours after the third injection, 0.5 μc of protein-bound Fe^{59} ferric citrate was injected; 19 hours later the uptake of Fe^{59} by the blood was measured in a scintillation counter with a 5-percent mean counting error. The erythropoietic activity was expressed as the percentage of Fe^{59} taken up, assuming the total blood volume of rats to be 5 percent of the body weight.

The response of the three groups of dogs differed in both time and magnitude of the apparent level of erythropoietin (Table 1).

The maximum response shown by the dogs in group III was attained on the second day; the level of erythropoietin had returned to normal by the 6th day. Group II showed no response until the 8th day, the uptake of Fe^{59} in the injected, fasted rats increasing to a maximum on the 12th day and declining slowly thereafter. In contrast, the dogs in group I showed a greater response than either group II or III, and the response was biphasic (Fig. 1). In the first phase, which began on the second day, the maximum uptake occurred on the 4th day; in the second phase, the maximum occurred on the 18th day. In magnitude, the second phase in the response of group I was comparable to that of the dog injected with phenylhydrazine; the serum of this dog, when assayed in ten rats, resulted in an Fe^{59} uptake of 13.27 $\pm$ 8.28 percent.

Table 1. The uptake of Fe^{59} in rats injected with sera from dogs that were irradiated and bled (group I); irradiated but not bled (group II); bled but not irradiated (group III). Each number represents the mean value from five rats $\pm$ standard deviation. Results obtained with untreated control rats were 3.71 $\pm$ 1.55; 3.78 $\pm$ 2.14; 3.00 $\pm$ 0.09; 2.68 $\pm$ 1.03; 3.36 $\pm$ 0.63 .

Day	Group I	Group II	Group III
0	3.03 $\pm$ 0.47	2.83 $\pm$ 0.03	2.68 $\pm$ 0.57
2	6.60 $\pm$ 2.46	3.61 $\pm$ 0.85	6.57 $\pm$ 1.23
4	12.43 $\pm$ 3.41	3.71 $\pm$ 0.72	4.94 $\pm$ 1.31
6	5.08 $\pm$ 1.71	2.98 $\pm$ 0.50	3.09 $\pm$ 0.93
8	5.17 $\pm$ 1.16	4.78 $\pm$ 1.36	2.71 $\pm$ 0.56*
12	5.98 $\pm$ 1.45	7.40 $\pm$ 2.75	3.06 $\pm$ 0.69*
13	7.85 $\pm$ 2.43	6.60 $\pm$ 0.49	2.67 $\pm$ 0.16
16	8.48 $\pm$ 2.91	5.44 $\pm$ 2.25	2.17 $\pm$ 0.35*
18	10.22 $\pm$ 2.19	4.88 $\pm$ 2.44	2.44 $\pm$ 0.25
21	9.87 $\pm$ 4.53		3.38 $\pm$ 1.05*

* One day before or after the indicated period.

Assuming that the assay method used here provides a reliable measure of the circulating level of erythropoietin, it is apparent that the irradiated dog is capable of producing erythropoietin in response to hemorrhage. It is significant that the dogs which were both irradiated and bled (group I) showed

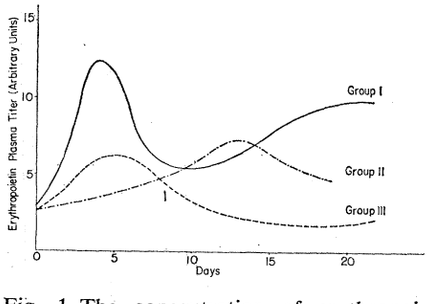

Fig. 1. The concentration of erythropoietin in the serum of dogs irradiated with gamma rays and then bled (group I); irradiated but not bled (group II); bled but not irradiated (group III).

an increase in the level of erythropoietin over the whole 3 weeks of the experiments. It thus seems reasonable to speculate that the rapid, persistent increase in the level of erythropoietin may favorably influence the recovery of bone marrow following irradiation, by providing an early and sustained stimulus to erythropoiesis.

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25 July 1963

Succinic Ester and Amide of Homoserine: Some Spontaneous and Enzymatic Reactions

Abstract. O-*Succinylhomoserine and* N-*succinylhomoserine have been synthesized. The first is rapidly transformed into the second by alkali. In acid, the second undergoes ring closure to the lactone, rather than the reverse acyl transfer. Neither supports the growth of methionine auxotrophs of* Neurospora *or* Salmonella. *However, bacterial extracts rapidly catalyze formation of a compound, chromatographically identical with cystathionine, from cysteine and* O-*succinylhomoserine. In the absence of cysteine the* O-*succinylhomoserine is converted to α-ketobutyrate. Both these reactions are absent from the same* Salmonella *mutant, and therefore are probably catalyzed by a single enzyme which is needed for methionine synthesis. Both reactions require pyridoxal phosphate.* N-*succinylhomoserine does not undergo either reaction.*

Rowbury has reported briefly on the biosynthesis of cystathionine from cysteine and homoserine in cell-free extracts of *Escherichia coli* (*1*). Successive incubations, in a given order, with extracts of different methionine auxotrophs revealed that at least two enzymes were required. The first converted homoserine and succinate, in the presence of adenosine triphosphate (ATP), coenzyme A (CoA), and glucose, into an intermediate which, together with cysteine and the second extract, yielded cystathionine. Subsequent evidence indicated that the intermediate was probably the succinic ester of homoserine (*1*). This clue to the major missing link in the pathway of bacterial trans-sulfuration was particularly interesting to us, since we had previously not been able, under a variety of conditions, to incorporate cysteine-S^{35} or homoserine-C^{14} into cystathionine with extracts of *Neurospora*. In contrast, formation of cystathionine by β replacement, from serine and homocysteine, could easily be shown in extracts of *Neurospora* and yeast, though this reaction was absent from *E. coli* and *Salmonella* (*2*). Recently we have synthesized the two obvious possible candidates for the intermediate containing succinate and homoserine, and we now report some studies of the chemical properties and enzyme-catalyzed reactions of these compounds.

For the preparation of *O*-succinyl-DL-homoserine (I), equimolar amounts of succinic anhydride and *N*-carbobenzoxy-DL-homoserine (*3*) were heated in dry pyridine until the reaction was complete, judged by the loss of neutral hydroxylamine and the persistence of alkaline hydroxylamine reactions. *O*-Succinyl-*N*-carbobenzoxy-DL-homoserine was purified as the amorphous dicyclohexylamine salt (mp 130° to 140°C). It was then converted to the free acid form (a liquid below 0°C), which was dissolved in glacial acetic acid for catalytic hydrogenolysis (*4*). Crude compound I was obtained in 88 percent yield by evaporation of the filtered solvent at reduced pressure. After several recrystallizations from aqueous-ethanol the melting point was 180° to 181°C. Analysis showed C, 43.65; H, 6.02; N, 6.54. The calculated values were C, 43.83; H, 5.98; N, 6.39.

N-Succinyl-DL-homoserine (III) was prepared by the addition, a little bit at a time, of excess succinic anhydride to DL-homoserine in aqueous NaOH (*5*), followed by elution through Dowex-50 H^+. After removal of succinic acid by crystallization, the glass-like residue was manipulated (for example, by warming in anhydrous solvents) until a second crystalline fraction was obtained in 25 percent yield which proved to be *N*-succinyl-DL-homoserine lactone (II). After recrystallization from a mixture of methanol and ethyl acetate the melting point was 142° to 143°C. Upon analysis there was found C, 46.84; H, 5.04; N, 7.11 (calculated: C, 47.76; H, 5.51; N, 6.96). The structures of both compounds were confirmed by infrared spectroscopy, and the spectrum of II was not compatible with its being the alternative lactone with a nine-membered ring (*6*).

Compound I gave the expected color yield with ninhydrin, but the alkaline (or neutral) hydroxylamine test for ester was completely negative. An explanation for this was suggested by the results of a titration. Titration of the acid form up to *p*H 12 showed the

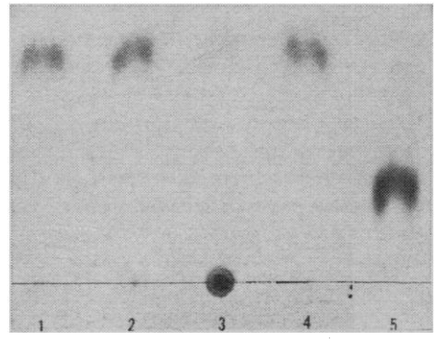

Fig. 1. Photograph of radiograms showing products formed after incubations of cysteine-S^{35} and homoserine derivatives with a dialyzed sound-treated extract of *E. coli* 26/18. All reaction mixtures (0.5 ml volume) contained (amounts in micromoles): potassium phosphate *p*H 7.5, 32; pyridoxal-phosphate, 0.03; L-cysteine-S^{35}, 6 (2 μc); enzyme, 2.5 mg. In addition there were the following supplements (since shown not to be required): ATP, 3; $MgCl_2$, 3; glucose, 6; L-cystathionine, 2. Reaction mixture No. 2 also contained DL-homoserine, 6, and succinate, 15. Reaction mixture No. 3 contained *O*-succinylhomoserine, 6. Reaction mixture No. 4 contained *N*-succinylhomoserine, 6. All mixtures were incubated 30 minutes at 37°C under helium. The mixtures were deproteinized, desalted, and treated with performic acid to oxidize cysteine and cystathionine. Portions of 5 percent of each were applied at the origin (1 to 4 on horizontal line) and chromatographed in a mixture of *t*-butanol, water, and formic acid (7:2:1). The R_f of cysteic acid corresponds to the faster radioactive spot. Oxidized cystathionine remains at the origin. The radioactive area at the origin, after chromatographing a larger portion of reaction mixture 3, was eluted with 2*N* HCl, and the eluate was rechromatographed in a mixture of propanol, 11*N* HCl and water (6:2:1). As shown in the area above No. 5 in Fig. 1 all of the radioactivity migrated to a single spot, which was again found in the same position as authentic oxidized cystathionine.