17070. Effect of Folic Acid, Liver Extract, and Vitamin B_{12} on Hemoglobin Regeneration in Chicks.*

C. A. NICHOL, A. E. HARPER,† AND C. A. ELVEHJEM.

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

No change in the blood hemoglobin level has been found to accompany the growth response of chicks to the injection of antipernicious anemia (APA) liver extracts[1] or vitamin B_{12}[2] when fed a corn-soybean oil meal basal ration containing adequate folic acid. The importance of folic acid for hemoglobin regeneration has been demonstrated with experimental anemias in several different species showing anemia and in the therapy of human macrocytic anemias. APA liver extracts have been found to be ineffective for the cure of anemia induced by folic acid deficiency in the chick[3,4] and in the rat.[5] Data presented here, based upon the rate of hemoglobin regeneration after production of a severe, chemically-induced anemia in folic acid depleted chicks, show that liver extracts or vitamin B_{12} are ineffective in increasing the rate of hemoglobin formation when given alone, but show a stimulatory effect when given with folic acid.

Experimental. Straight-run (New Hampshire ♂♂ x Single Comb White Leghorn ♀♀) crossbred chicks which were the progeny of hens fed diet B-1 described previously[6] were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The chicks were wing-banded when one day old. Weights were recorded at weekly intervals during the depletion period.

All chicks received a folic acid deficient purified ration containing dextrin 61, alcohol-extracted casein 18, gelatin 10, salts V 6,[7] soy oil 5, l-cystine 0.3, and fortified haliver oil (60,000 U.S.P. units of vitamin A, 6,000 A.O.A.C. units of vitamin D_3 per g) .04 g; thiamin 0.3, riboflavin 0.6, nicotinic acid 5.0, pyridoxine 0.4, calcium pantothenate 2.0, biotin 0.02, choline chloride 150, inositol 100, 2-methyl-1,4-naphthoquinone 0.05 and α-tocopherol 0.3 mg.

Chicks fed the folic acid deficient, purified ration developed a slight anemia in 14 to 24 days. Hemoglobin levels varied widely, but the decrease averaged 1 to 2 g below normal. At the end of this depletion period a single intramuscular injection of phenylhydrazine hydrochloride (2.0 mg per 100 g body weight) was given. Severe anemia developed within 2 days (Hb range, 1-5 g %). Reagent grade phenylhydrazine hydrochloride was treated with charcoal and recrystallized from hydrochloric acid solution. An aqueous solution (10 mg per cc) was injected into the pectoral muscle using a 1 cc calibrated hypodermic syringe.

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† Present address: Department of Biochemistry, University of Alberta, Edmonton, Canada.

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Comparable groups of chicks having hemoglobin values ranging from 2 to 4 g % were then arranged. Experimental treatments were followed by determining hemoglobin levels at 2 to 4 day intervals, using the alkaline oxyhemoglobin method[8] as adapted for the Evelyn colorimeter.[9] Blood samples were taken directly by puncture of a wing vein.

Dosages of phenylhydrazine hydrochloride from 1 to 6 mg per 100 g body weight were tested. 2.0 mg was selected as the smallest dosage which caused a suitable decrease in hemoglobin level within 2 days. It was also observed that a rapid return to normal hemoglobin level occurred if the ration contained adequate folic acid. The hemoglobin level of normal chicks dropped to about 5 g % within 2 days after the injection of phenylhydrazine hydrochloride. Three to 4 days following this anemic condition, their hemoglobin level had returned to normal. Consequently, small amounts of folic acid must be given to the folic acid depleted, anemic birds to prolong the period of recovery so that differences in rate of hemoglobin regeneration can be measured.

Experiment 1. Four groups of deficient chicks, having hemoglobin values of 2 to 4 g %, were selected on the 16th day (2 days following the injection of 2.0 mg of phenylhydrazine hydrochloride per 100 g body weight). Six chicks were used in each group and no losses occurred during the following treatments (1) control group, untreated, (2) liver extract (Lilly, reticulogen, 20 U.S.P. units per cc), 1 unit per bird, injected, (3) folic acid, 20 γ per bird, injected, (4) liver extract, 1 unit per bird and folic acid, 20 γ per bird, injected. The dosages were given on the 16th, 18th and 20th days.

The changes in the hemoglobin values following the above treatments are shown in Fig. 1. Liver extract alone did not influence the rate of hemoglobin regeneration. By the 24th day, the hemoglobin values of the groups receiving folic acid and folic acid in combination with liver extract had returned to

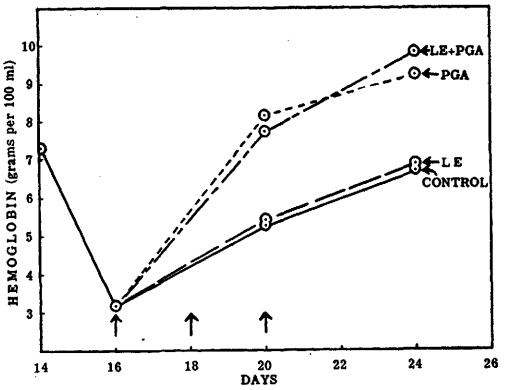

FIG. 1.
Rate of Hemoglobin Regeneration in Anemic Chicks.
The control group was untreated. In the other groups each bird received: LE—1 unit liver extract; PGA—20 γ folic acid; LE + PGA—1 unit liver extract and 20 γ folic acid, injected on the 16th, 18th, and 20th days.

normal and did not differ significantly from each other. The least significant difference between treatments on the 24th day was 1.23 g %.

Exp. 2 The pattern of Exp. 2 was similar to that used in Exp. 1 except that the depletion period was extended to 21 days before injection of phenylhydrazine hydrochloride. Pteroylheptaglutamic acid and pteroyltriglutamic acid were tested at equimolar dosage to pteroylglutamic acid (PGA). All of the 5 chicks used in each test group survived.

As shown in Fig. 2, liver extract alone did not increase the rate of hemoglobin regeneration. PGA alone did. The triglutamate and heptaglutamate forms were as active as PGA. Liver extract with folic acid resulted in more rapid regeneration of hemoglobin than was observed on folic acid alone. A significant difference in the average hemoglobin values of these two groups was noted on the 29th day and again on the 31st and 33rd days. The least significant difference between treatments by the 33rd day was 1.27 g %.

Exp. 3. Chicks were depleted for 24 days on a folic acid deficient, purified ration in which sucrose was used as the source of carbohydrate. In addition to the treatments used in Exp. 1 and 2, pure vitamin B_{12} was tested alone and in combination with folic acid. In the control group, 5 of the 8 birds

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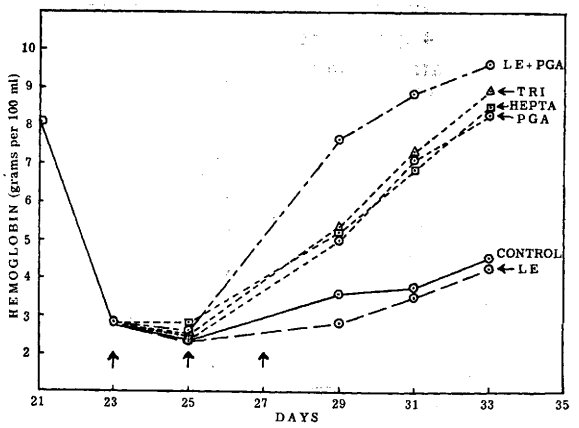

FIG. 2.

Rate of Hemoglobin Regeneration in Anemic Chicks. The control group was untreated. In the other groups each bird received: LE—1 unit liver extract injected on the 23rd, 25th, and 27th days; HEPTA—pteroylheptaglutamic acid and TRI—pteroyltriglutamic acid at equimolar dosage to PGA—pteroylglutamic acid, 5 γ, 10 γ, and 20 γ injected on the 23rd, 25th, and 27th days respectively; LE + PGA—1 unit liver extract and 5 γ folic acid injected on the 23rd day, 1 unit liver extract and 10 γ folic acid injected on the 25th day, 1 unit liver extract and 20 γ folic acid injected on the 27th day.

survived on the 38th day and 3 survived beyond the 42nd day. At least 7 of the 8 chicks survived beyond the 42nd day in each of the other test groups.

The average hemoglobin values for each group are shown in Fig. 3. The pattern of hemoglobin regeneration induced by vitamin B_{12} was the same as that observed with liver extract. Vitamin B_{12} alone had no greater effect than liver extract, yet in combination with folic acid the rate of hemoglobin regeneration was more rapid than that resulting from treatment with folic acid alone. By the 42nd day, the least significant difference between treatments was 1.20 g % hemoglobin.

In addition to the group shown in Fig. 3 which received 1 γ of vitamin B_{12} plus 10 γ folic acid on the 26th and 29th days, a second group of 8 chicks which received 0.1 γ of vitamin B_{12} plus 10 γ of folic acid on these days showed an average hemoglobin value of 8.24 g % on the 42nd day and another group which received double the dosage of folic acid alone (20 γ) on these days had an average hemoglobin value of 7.71 g % on the 42nd day.

Discussion. A series of 5 experiments in all were carried out, each of which included the 4 experimental treatments outlined in Exp. 1. Liver extracts used in the treatment of pernicious anemia were not effective in stimulating the rate of hemoglobin regeneration in anemic chicks depleted of folic acid. In each of the 4 experiments having a depletion period of 21 days or longer, the rapid rate

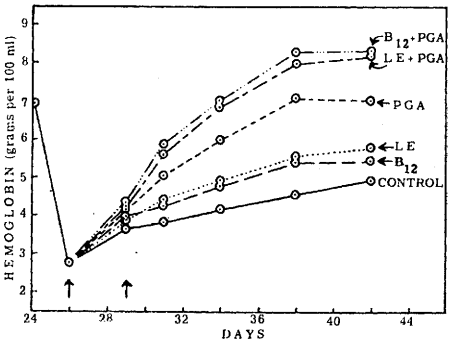

FIG. 3.

Rate of Hemoglobin Regeneration in Anemic Chicks.

The control group was untreated. In the other groups each bird received: LE—1 unit liver extract; B_{12}—1 γ vitamin B_{12}; PGA—10 γ folic acid; LE + PGA—1 unit liver extract and 10 γ folic acid; B_{12} + PGA—1 γ vitamin B_{12} and 10 γ folic acid, injected on the 26th and 29th days.

of hemoglobin regeneration following treatment with liver extract and folic acid was significantly greater than the rate observed following treatment with an equal dosage of folic acid alone. Less than 1 γ of folic acid was present in the 1 U.S.P. unit of liver extract injected. The component causing the stimulation of hemoglobin regeneration in the presence of folic acid appears to be vitamin B_{12}.

The metabolic interrelationship between folic acid and vitamin B_{12} is still obscure. Since in the anemic chicks, folic acid alone in large amounts will bring about rapid and complete return to normal hemoglobin level, the observed stimulation of hemoglobin regeneration may be due to an indirect action of vitamin B_{12} which makes more folic acid available to the animal organism. Vitamin B_{12} may also act directly in the normal mechanism of hemoglobin formation. This is implied by the activity of vitamin B_{12} in cases of pernicious anemia. The chicks used in these studies, like those used for the growth assay of vitamin B_{12},[2] were depleted by control of the hen's diet. Under the special conditions described, the stimulation of hemoglobin regeneration may be a demonstration of the direct role of vitamin B_{12} in the mechanism of hemoglobin formation. This work emphasizes that folic acid and vitamin B_{12} may have distinct and separate functions and indicates that vitamin B_{12} does not have optimum activity unless folic acid is present in adequate amounts.

The effectiveness of the technic is demonstrated by the similar response to the three forms of folic acid shown in Fig. 2. In each experiment, analysis of variance was applied on the last day. This is a more severe test of significance, since individual variation becomes greater as the interval following treatment increases. For example, in experiment 2 the least significant difference between treatments on the 31st day was 1.05 g % hemoglobin. By the 33rd day the least significant difference had increased to 1.27 g %.

Phenylhydrazine hydrochloride is used in the therapy of polycythemia vera. The anemia induced is due to hemolysis of the mature red blood cells, but the mechanism is not known.[10] Yeshoda and Damodaran[11] used a phenylhydrazine induced anemia in rats in a study of the influence of tryptophan on hemoglobin formation. The application of this technic to folic acid depleted chicks increases the range over which differences in hemoglobin levels can be measured and permits selection within narrow limits of comparable groups of chicks having a severe anemia.

Summary. A severe anemia was induced in chicks by the intramuscular injection of phenylhydrazine hydrochloride (2.0 mg per 100 g body weight) following a depletion period on a folic acid deficient purified ration. Chicks were selected within narrow limits (2-4 g % Hb) and arranged into comparable groups to study the effect of folic acid, liver extract and vitamin B_{12} on the rate of hemoglobin regeneration.

Liver extract alone did not influence the rate of hemoglobin formation. When the depletion period was 21 days or longer, the combination of liver extract and folic acid caused a more rapid regeneration of hemoglobin than treatment with a similar dosage of folic acid alone. Pure vitamin B_{12} completely replaced liver extract in stimulating the formation of hemoglobin in the presence of folic acid.

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