DIETARY FACTORS CONCERNED IN ERYTHROPOIESIS

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TABLE OF CONTENTS

I. Introduction

II. Vitamins: A. Riboflavin; B. Nicotinic Acid; C. Pyridoxine; D. Lactobacillus Casei Factors; E. Extrinsic Factor; F. Ascorbic Acid; G. Other Vitamins

III. Amino Acids: A. General Protein Deficiency; B. Composition of Globin; C. Role of Amino Acids

IV. Minerals: A. Iron; B. Copper; C. Cobalt

V. Discussion To appear in

VI. Summary

1. INTRODUCTION

Knowledge concerning the formation of the erythrocyte is far less widespread than that concerning its destruction. The breakdown through the various bile pigments has been extensively studied and several of the intermediary compounds have been isolated and identified. Although hemin has been synthesized in vitro by Fischer and Zeile there is little evidence to indicate that such steps take place in vivo. The intermediary compounds have not been isolated, and this approach has not been productive. However, it is known that the absence of certain dietary essentials retards erythrocyte formation and the addition of these essentials to the diet accelerates it. It seems hopeful that a study of the dietary factors concerned in the formation of the erythrocyte will define the building stones and in time allow them to be pieced together so as to afford an understanding of the subject. It might be expected that as each factor is clearly defined, relationships will be discovered and the processes of hemoglobin formation and erythropoiesis will unfold. It is the purpose of this review to describe those dietary factors necessary for erythropoiesis which are now recognized and to discuss the present knowledge concerning them.

The known factors concerned in the formation of the red cell may be divided into three groups: vitamins, amino acids, and minerals. It might be supposed that these substances could be divided into those concerned with the formation of the stroma of the red cell and those concerned directly with the formation of hemoglobin. At the present time it is impossible to make this division, and it will be necessary to consider the formation of the erythrocyte and hemoglobin as one process, erythropoiesis. The term "hemoglobin formation" has been used here loosely, as in the literature, to refer to erythropoiesis.

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Because of the enormous volume of literature on the subject much of it has had to be omitted. Much of the earlier work in which complex diets and crude extracts were used is inconclusive and for this reason has been omitted. Data in regard to the type of anemia produced have had to be reviewed critically. Although much of the nutritional work has been carefully done detailed hematological studies have been neglected. Studies of the cell size and of the concentration of the hemoglobin in the cells have many times not been made. The terms "hypochromic," "normochromic," and "hyperchromic" have been used in the literature loosely. These terms are employed in this review only when referring to studies in which the mean corpuscular hemoglobin concentration was calculated. In general, only that work which is clearly defined has been reviewed.

The nutritional, chemical, and physiological approach to the formation of the red blood cells is relatively new and views are ever changing. With each change certain concepts are proved false, new ones are added, and others are more firmly established. This is both natural and desirable. It is a dynamic field for investigation.

II. VITAMINS

It has long been suspected that vitamins play a role in red blood cell formation but it was not until "synthetic" diets were available that a specific vitamin deficiency could be studied. It has now been definitely shown that riboflavin, nicotinic acid, pyridoxine, and the various "folic acids" are important for red blood cell formation in at least one species each. The role of ascorbic acid, pantothenic acid, choline, and biotin in erythropoiesis is not clearly established and there is no substantial evidence that thiamine, p-aminobenzoic acid, or inositol is concerned.

A. Riboflavin.—The role of riboflavin in blood regeneration in dogs on a purified ration has been carefully studied by Spector, Maass, Michaud, Elvehjem, and Hart. A mild anemia developed on the purified ration without riboflavin and a severe anemia was readily induced with slight bleeding. In spite of cessation of phlebotomy the dogs were unable to recover from the anemia. The average weekly production of hemoglobin was only 2 to 6 grams. The animals showed good hemoglobin regeneration and recovered rapidly from the anemia when 30 micrograms per kilo of body weight per day was administered per day. Only a slight and variable hemoglobin production was observed below a level of 15 γ per kilo of body weight per day in growing dogs. The adult dog was able to show good hemoglobin regeneration at a level of 15 γ per kilo daily. During the depletion period the anemia produced was of the microcytic hypochromic type. If riboflavin was given but phlebotomy was carried out, a decrease in hemoglobin, hematocrit, and erythrocyte count took place but the anemia was normocytic hypochromic in type. From this the authors conclude that riboflavin plays a role in determining the size of new red cells.

Wintrobe, Bushke, Follis, and Humphreys have reported the development of anemia in two riboflavin-deficient pigs which survived a long time. The anemia...
was moderate, gradually progressive, and normocytic. Leukopenia was not marked although the leukocyte count at the time of death was low as compared with the initial values. Demyelinization of the brachial and sciatic nerves was found in one animal. Spinal cord changes were not noted.

A moderate impairment in the rate of red blood cell and hemoglobin regeneration in the rat as a result of riboflavin deficiency has been demonstrated. Anemia was noted in a few riboflavin-deficient rats not subjected to hemorrhage. Waisman has reported that monkeys maintained on a highly purified diet consisting of pure vitamins except riboflavin, together with a folic acid concentrate, develop an unmistakable anemia. Maximal anemia developed in 59 to 108 days. The red cell counts fell from a level of 5 to 6 million down to 2 to 3 million and the hemoglobin from 13 to as low as 4 grams. The reduced red cell count usually preceded the decrease in hemoglobin. There was also a definite lowering of the white blood cell count. In a more recent paper from the same laboratory the presence of anemia in monkeys on a riboflavin-deficient diet was confirmed. Determinations of the cell size and corpuscular hemoglobin concentration were not done. Upon administration of riboflavin there was a definite increase in hemoglobin, red cell and white cell count in the blood.

A relationship between riboflavin and erythropoiesis was suggested but not definitely proved in the earlier literature. Johnstone and Reed reported that monkeys maintained on riboflavin-deficient diets developed a macrocytic anemia, hypochlorhydria, and leukopenia. Their diet, however, was deficient in all other members of the B complex except thiamine. Hemorrhagic anemia in rabbits was reported as responding in part to riboflavin administration. Earlier work in the dog failed to demonstrate an anemia but myelin degeneration of peripheral nerves and posterior columns of the spinal cord was reported. Sebrell and Onstott reported that dogs maintained on a diet of polished rice, casein, sucrose, corn, cod liver oil, and salt mixture developed a "hypochromic" anemia with hemoglobin values ranging between 7 and 13 grams per cent. The bone marrow was reported as being largely fatty. However, there was no constant and material improvement in the blood picture following the administration of large doses of riboflavin, and it is quite likely that the anemia observed was due to pyridoxine deficiency rather than to riboflavin deficiency. In 1933 Miller and Rhoads reported that dogs maintained on a modified Goldberger "black tongue"-producing diet developed glossitis, stomatitis, diarrhea, a definite macrocytic anemia, and a hyperplastic megaloblastic bone marrow not unlike that seen in sprue and pernicious anemia. Since riboflavin was not given to these animals to test the response of the blood it was not demonstrated that the changes were due specifically to riboflavin deficiency. These animals were also deficient in other vitamins. More recent work using purified diets has failed to confirm the presence of a macrocytic anemia. György, Robscheit-Robbins, and Whipple found that the administration of a synthetic riboflavin to standardized anemic dogs produced a definite increase in hemoglobin production above the basal level. Potter, Axelrod,
and Elvehjem found that dogs on a highly purified diet deficient only in riboflavin developed a mild anemia with hemoglobin values ranging between 9.9 and 12.3 Gm. per cent.

There is no substantial evidence that riboflavin deficiency in human beings results in anemia. Gobell obtained increases in the red blood cell count of premature infants by the administration of nicotinamide and riboflavin. Sebrell and Butler maintained 18 patients for 335 days on a riboflavin-deficient diet and found no significant change in the hemoglobin or red and white blood cell counts. Moore et al. found that the hypochromic anemia occurring in patients with ariboflavinosis responded favorably and completely to iron therapy alone. Since myelin degeneration of the peripheral nerves and posterior columns of the spinal cord occurs in riboflavin-deficient dogs it was suggested that this vitamin might play a role in the etiology of subacute combined degeneration of the cord. However, the absorption and excretion of riboflavin has been shown to be normal in patients with pernicious anemia and therapy with this vitamin is ineffective. That riboflavin is not the extrinsic factor has been conclusively demonstrated.

Table 1.—Hematological Findings in Chronic Nicotinic Acid Deficiency in Dogs. Modified After Handler and Featherston

<table>
<thead>
<tr>
<th>State</th>
<th>R.B.C. Millions per cu. mm.</th>
<th>Hemoglobin Gm. per 100 cc.</th>
<th>Hematocrit per cent</th>
<th>Mean Corpuscular Volume micra</th>
<th>Mean Corpuscular Hemoglobin mcg.</th>
<th>Mean Corpuscular Hemoglobin conc. per cent.</th>
<th>Reticulocytes per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6.9</td>
<td>15.2</td>
<td>45.5</td>
<td>66</td>
<td>12</td>
<td>33</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Anemic</td>
<td>2.8</td>
<td>6.8</td>
<td>25.1</td>
<td>89</td>
<td>14</td>
<td>17</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Normal</td>
<td>7.2</td>
<td>16.6</td>
<td>51.4</td>
<td>71</td>
<td>13</td>
<td>31</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Anemic</td>
<td>3.3</td>
<td>5.5</td>
<td>16.9</td>
<td>74</td>
<td>14</td>
<td>32</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Klein and Kohn have shown that the synthesis of flavinadenine dinucleotide from riboflavin takes place in human blood cells both in vitro and in vivo. It seems possible that this enzyme may be related to erythropoiesis or hemoglobin synthesis. It is known that d-amino acids are deaminized by an enzyme which contains the flavinadenine nucleotide, and Spector et al. have suggested that this vitamin may be concerned in the metabolism and arrangement of the amino acids of globin.

B. Nicotinic Acid.—Anemia due to nicotinic acid deficiency was obscure and indefinite until Handler and Featherston demonstrated that the parenteral administration of physiological saline solution to dogs with acute black tongue resulted in alleviation of the existing hemoconcentration and was associated with the appearance of severe anemia (table 1). Determination of total blood volume and total circulating hemoglobin revealed a reduction of 86 per cent in the total circulating hemoglobin. Thus, since the total blood volume was greatly reduced the anemia was actually more severe than it appeared to be. The total hemoglobin content of the normal dogs averaged 28.4 grams while the nicotinic acid-deficient animals possessed an average of only 39 grams of hemoglobin. The anemia fell into
two definite groups in respect to cell size. In one group it was macrocytic with an increase in the mean corpuscular volume from 66 to 89 cu. microns. The mean corpuscular hemoglobin concentration declined in proportion to the increase in cell size. In the second group the anemia was normocytic and normochromic. There were no instances of microcytosis. Gastric analyses were performed on 14 dogs and in no case was there any apparent impairment of acid secretory function. There was no reticulocytosis accompanying the anemia and the total serum bilirubin was not elevated. The anemia failed to respond to the administration of iron, protein, glucose, hemoglobin, antipernicious anemia factor, xanthopterin, and cobalt. Following the administration of nicotinic acid or nicotinamide there was an immediate reticulocyte response which reached a maximum of 15 to 30 per cent after 3 to 4 days and subsided in about 10 days. The red cell counts, hemoglobin, and volume of packed red cells continued to rise following the reticulocytosis and reached normal in 30 to 40 days. The red cells returned to their original size in every instance.

Histological examination of the tissues of one dog was made and revealed hemosiderosis of the spleen. The femur marrow contained hemosiderin granules and in addition appeared "exhausted." The cells of the leukocytic series were reduced in number and there was little erythropoietic tissue. Erythropoiesis appeared to have stopped at the erythroblast level. These authors postulated that since immature nucleated erythrocytes respire, they probably use pyridine nucleotides in this respiration. As the supply of nicotinic acid diminishes, anemia develops owing to lack of cozymase in the earliest stages of cell development. Thus, in dogs, nicotinic acid deficiency results in a severe anemia which is either macrocytic normochromic or normocytic normochromic and is accompanied by a hypoplastic bone marrow. The anemia responds to nicotinic acid therapy.

Nutritional pancytopenia in rats has been reported as being prevented by nicotinic acid. This has not been confirmed.

The association of anemia with pellagra has long been known. Anemia is reported in various series to be present in 44, 63, and 84 per cent of the cases. It is variable in nature. Huck, Boggs and Padget, and Mollow and Klein observed a "secondary" type of anemia in all of their cases except one. Turner in a careful study of 22 anemic pellagrins found that the anemia was either normocytic or microcytic and in no instance was it macrocytic in type. Thirty-four per cent had erythrocytes with corpuscular hemoglobin concentration less than normal, while in 66 per cent the concentration was within normal range. Spies and Chinn in a study of 30 severe "alcoholic" pellagrins found that in 75 per cent there was a definite increase in the volume of the red blood cells and a color index of one or above. In the other 25 per cent there was an anemia which was characterized by a decrease in the red blood cell volume and color index. Moore, Vilter, Minnich, and Spies have reported the occurrence of macrocytic anemia in 56 patients who had existed for years on diets inadequate in animal protein and in the vitamins of the B complex. Most of the patients had clinical evidence of pellagra, ariboflavinosis, or beriberi. Niacin given in conjunction with 7 other known synthetic B vitamins did not affect the erythropoietic equilibrium. Moore,
MINNICH, VILTER, AND SPIES in a study of hypochromic anemia in 32
patients with pellagra and other vitamin deficiencies reported that iron therapy alone produced a
satisfactory reticulocyte response and rate of hemoglobin regeneration. Thus, there
is no evidence that the anemia associated with pellagra is due to nicotinic acid
deficiency. Proof that such an anemia exists in man is lacking.

Gastric achlorhydria occurs in approximately 80 per cent of the patients with
pellagra. However, it has been demonstrated that the intrinsic factor is present
in the gastric secretions from pellagrins in an amount adequate to form the anti-
anemic substance. That nicotinic acid is not the extrinsic factor of Castle has
also been adequately demonstrated.

It is now generally agreed that an increased excretion of porphyrin in the urine
is not an essential feature of pellagra and when porphyrinuria appears it is most
likely due to some alteration of liver function.

C. Pyridoxine.—The development of severe anemia has been adequately demon-
strated to result from pyridoxine deficiency in dogs and swine. A mild
anemia develops in pyridoxine-deficient chicks and in this species the anemia is
accompanied by a decreased clotting time, hyperprothrombinemia, and small
spleens. The earlier reports on pyridoxine-deficient rats either failed to reveal the
presence of anemia or at most only a slight anemia was noted. KORNBERG, TABOR,
and SEBRELL have recently studied blood regeneration in pyridoxine-deficient rats
in detail. A moderately severe anemia was found in 18 per cent. Granulocytopenia
was present in 4 of these. In 15 per cent a mild anemia was noted. No anemia was
found in 67 per cent. A latent erythropoietic inadequacy indicated by an impair-
ment in the rate of red blood cell regeneration after hemorrhage was demonstrated
in all pyridoxine-deficient rats.

Morphologically the anemia in dogs and swine is microcytic and slightly
hypochromic. In dogs there is a reduction of the mean corpuscular volume from
approximately 70 to 48 cubic microns. The mean corpuscular hemoglobin concen-
tration is slightly reduced. In swine the mean corpuscular volume is reduced to an
average of 38 cubic microns from an average of 58 and the mean corpuscular hemo-
globin concentration changes from 33 per cent to 29 per cent. The anemia is severe
in both species, the hemoglobin falling as low as 1.4 grams per 100 cc. of blood.
In pigs a volume of packed red cells as low as 9 cc. per 100 cc. of blood has been
reported. A significant anemia develops in either species within two months. In
pigs, as anemia develops anisocytosis becomes marked but poikilocytosis is rare.
The "Price-Jones" curve of distribution of the red cell diameters is "shifted to the
left" and the mean diameter is reduced. Large polychromatic red corpuscles make
their appearance but are outnumbered by the microcytes. An irregular reticulo-
cytosis as great as 10 to 12 per cent may appear. A large number of red corpuscles
are seen to contain a single, round and moderately large, blue-staining granule
resembling a nuclear particle. Normoblasts may be increased to as many as 4 and
even 8 per 100 leukocytes.

The bone marrow in pyridoxine-deficient swine is hyperplastic. The femoral
marrow is cellular rather than fatty and there is a definite increase in nucleated red
blood cells as well as in undifferentiated "blast" cells. Following therapy with crystalline pyridoxine the marrow becomes normoblastic. Information concerning the marrow of pyridoxine-deficient dogs is scant. Fouts et al.26 made such an examination in only one animal, and in this animal the marrow was red and hyperplastic and consisted chiefly of normoblasts. In the one animal studied after therapy the bone marrow was normal.

Hemosiderosis of the spleen, liver, and bone marrow has been reported in swine.44 There is a great accumulation of hemosiderin in the pulp of the spleen which is both intra- and extra-cellular but the granules are conspicuously absent from the malpighian bodies. Following therapy the hemosiderosis of the liver and bone marrow disappears and that in the splenic pulp diminishes. By restricting the intake of iron the hemosiderosis can be entirely prevented.14 Hemosiderosis in pyridoxine-deficient dogs has not been reported.

Ataxia and convulsions have been reported to develop in both dogs43 and swine42-45 and pathologically the nervous system shows interesting changes. Degeneration takes place in the peripheral nerves, the spinal ganglia, the posterior roots, and the dorsal funiculi of the spinal cord. It would seem that pyridoxine, like the antipernicious anemia substance and copper, is essential to the integrity of both the nervous and hematopoietic systems.

There is no evidence for the presence of a hemolytic element in pyridoxine deficiency anemia. McKibbin, Shaefer, Frost, and Elvehjem13 reported that the plasma bilirubin level was normal in dogs deficient in pyridoxin. Cartwright, Wintrobe, and Humphreys46 studied serum bilirubin, per cent reticulocytes in the blood, icterus index, quantitative urobilinogen excretion in the stool and urine, and urinary excretion of porphyrin in swine and compared this anemia with that induced by phenylhydrazine. They could find nothing to indicate that an increased rate of hemolysis occurs in pyridoxine deficiency.

Elevated plasma iron levels in dogs were reported by Fouts et al.39 and confirmed by McKibbin et al.11 The serum iron which is normally around 100 γ per cent rises to the extremely high values of 300 to 500 γ per cent. The serum iron increase has been studied in detail in swine.46 The increase begins at approximately the 4th week of the deficiency and reaches its maximum (350 to 600 γ per cent) between the 5th and 10th weeks. Following this the serum iron level tends to decline somewhat. Combined iron and pyridoxine deficiency results in an abnormally low serum iron level and in this combined deficiency hemosiderosis of the tissues is prevented. It would seem, then, that the ferremia of pyridoxine deficiency is caused by continued retention of iron at a time when its utilization for hemoglobin synthesis is at a minimum. The possibility exists that the absorption of iron is increased in pyridoxine deficiency.

Studies on the nature of the iron in pyridoxine deficiency reveal that approximately 95 per cent of the increased iron in the serum is in a nondialyzable, ferric state.16

Studies on the whole blood copper levels in dogs anemic because of pyridoxine deficiency reveal that this element is present in the low normal range and that
there is a slight rise after treatment with pyridoxine. This would seem to indicate that copper, unlike iron, is not mobilized in the blood of the severely anemic animal.

Since it is possible that pyridoxine is essential for the synthesis of hemoglobin, an iron-porphyrin complex, Lepkovsky and Parsons studied the effect of pyridoxine deficiency on the synthesis of another iron-porphyrin complex, namely, catalase, and found that there was no significant change in the catalase activity of the liver, kidney, and heart tissues of the rat. Thus there is no evidence to date that pyridoxine is directly concerned with the synthesis or metabolism of the porphyrins.

It has been shown that a relationship exists between pyridoxine and tryptophan metabolism. Pyridoxine-deficient rats, mice, dogs, and swine excrete in the urine abnormally large quantities of xanthurenic acid, a metabolite of tryptophan metabolism. The amount excreted is related to the amount of l-tryptophan ingested. It seems that as the protein intake is increased the symptoms of the deficiency become more severe and the survival time is diminished. Since a deficiency of either pyridoxine or tryptophan gives rise to anemia, the question arises as to whether the two substances combine to form a third which is essential to blood formation. If this were true then a deficiency of either one should result in the same morphologic changes in the blood. This cannot be the case since the anemia of tryptophan deficiency is normocytic, it is accompanied by a terminal leukopenia, the serum iron level is normal, hypoproteinemia exists, the bone marrow is normo- or hypoplastic and there is no hemosiderosis.

In swine, combined pyridoxine and iron deficiency results in a greater anemia than does either deficiency alone. The combined deficiency also results in a lower mean corpuscular volume. In one animal with the combined deficiency the hemoglobin reached 2 Gm. per cent and the mean corpuscular volume diminished to 7.4 cu. μ (normal 8). The anemia of pyridoxine deficiency responds to treatment with synthetic pyridoxine hydrochloride. A reticulocytosis develops reaching a peak in two to six days and the reticulocytes may be increased to as much as 30 per cent. Following the reticulocytosis there is rapid restoration of the hemoglobin, volume of packed red cells, and mean corpuscular volume toward normal. The magnitude of the changes in the blood following pyridoxine therapy appears to be related to the size of the dose given, the degree of anemia, and the route of administration. The highest reticulocyte increases and degrees of increase in hemoglobin and volume of packed red blood cells in pyridoxine-deficient swine were observed when the anemia was most severe and large amounts of pyridoxine were given intravenously. Doses as small as 10 and 20 μg. per kilogram of body weight by mouth daily are ineffective in swine. A dose of 80 μg. per kilogram of daily weight by mouth daily is followed by a definite and pronounced response.

Many investigators have reported that although the anemia responds to pyridoxine, the addition of this factor alone to a synthetic diet is not sufficient to maintain the hemoglobin at optimal levels. There is at least one factor
in addition to pyridoxine which is found in liver and brewer's yeast and which is necessary to maintain hemoglobin production. The nature of this factor or factors is not known. It is not nicotinic acid, thiamine, riboflavin, pantothenic acid, choline, or inositol.

It has been demonstrated that the anemia does not respond to iron administration, either oral or intravenous, or to copper, cobalt, chlorophyll, chlorophyllin, concentrated liver extract, tryptophan, corn oil, hemoglobin, or hemin. Two derivatives of pyridoxine, pyridoxal, the 4-aldehyde of pyridoxine, and pyridoxamine, the 4-amine of pyridoxine, have been shown to be 5500 and 8000 times as active, respectively, in promoting the growth of *Streptococcus lactis* R as pyridoxine. The relative value of these derivatives in the prevention and treatment of the anemia has not been studied.

It has been shown that pyridoxine, pyridoxal, and pyridoxamine function as the coenzyme of tyrosine, lysine, arginine, and glutamic acid decarboxylase. The synthetic codecarboxylase prepared from pyridoxal has been found to be active with 3 of these enzymes. From these data it has been suggested that one of the functions of the vitamin B<sub>6</sub> group is to function as a coenzyme of amino acid decarboxylases. The relationship between this function and erythropoiesis is not obvious. It has been both asserted and questioned that pyridoxine plays a role in the biological transamination reactions. Recently it has been rather conclusively shown that pyridoxal phosphate functions as the coenzyme of the glutamate-aspartate transaminase. The function of the vitamin B<sub>6</sub> group in protein metabolism is therefore at least partially explained by its action in amino acid decarboxylation and transamination.

Pyridoxine has never been conclusively demonstrated to be essential to human nutrition. Pyridoxine deficiency anemia resembles pernicious anemia in several respects. In both conditions there is an increase in serum iron, hemosiderosis of the tissues, hyperplastic bone marrow, and neurological lesions. That they are not the same is evidenced by a microcytosis in one and a macrocytosis in the other. Pyridoxine anemia does not respond to liver extract and it has been shown that this vitamin is not the extrinsic factor. Pyridoxine anemia possesses some of the features of Mediterranean anemia. In both the red corpuscles are microcytic and hypochromic, serum iron is elevated, and iron-containing pigment is found in the tissues. Both types of anemia fail to respond to iron therapy. Goldman and Malavazos have reported that pyridoxine is of value in the treatment of this anemia when given in conjunction with pregnancy-urine hormone. The present author studied the effect of pyridoxine alone on the same patients reported by Goldman and Malavazos and was unable to demonstrate an effect on either the serum iron or the anemia.

Kark, Lozner, and Meiklejohn treated 4 cases of pellagra, 1 case of "idiopathic" hypochromic anemia, and 1 case of nutritional macrocytic anemia with pyridoxine, and in none of their cases did the anemia respond to pyridoxine therapy. Moore, Minnich, Vilter, and Spies studied 32 patients with hypochromic microcytic anemia associated with nutritional deficiency of various types and found that iron therapy alone produced a satisfactory reticulocyte response.
and rate of hemoglobin generation. Brewer’s yeast had no demonstrable effect in increasing the efficacy of iron therapy. They concluded that if pyridoxine deficiency was present in these patients it was not accompanied by hypochromic anemia with high serum iron levels. It has also been our experience that the serum iron level is low rather than high in deficiency states. However, if an associated iron deficiency is present this would be expected since a combination of the two deficiencies in the experimental animal results in a low rather than a high serum iron level.

In our experience pyridoxine has been found to be ineffective in the treatment of anemia of nephritis, aplastic anemia from various causes, the anemia of infections, pernicious anemia, Mediterranean anemia, and the anemia of malnutrition.72 Cantor and Scott7' have reported 3 cases of agranulocytic angina of toxic origin which responded to the intravenous administration of pyridoxine hydrochloride. They suggest that pyridoxine acts by direct stimulation of the myelocytic elements of the bone marrow. Fishberg and Vorzimer75 have reported that pyridoxine brings about a rapid and significant rise in the number of circulating granulocytes in human beings after a depression caused by thiouracil. These results are rather surprising since the white cells seem to be unaffected in experimental pyridoxine deficiency. Since granulocytosis may disappear spontaneously, especially when the offending agent is removed, acceptance of these claims concerning the value of pyridoxine in agranulocytosis must await further evidence.

D. Lactobacillus Casei Factors.—The various new factors described as having growth activity for Lactobacillus casei and related organisms are many. All of these factors have been found to have hemopoietic activity for one or more species of animals under certain conditions. Recently considerable clinical interest has developed in this group of substances because of the demonstration of the effectiveness of the synthetic L. casei factor in various macrocytic anemias in relapse. The early research in this field illustrates magnificently the importance to clinical medicine of so-called “ultrascientific” research. A recent review by Berry and Spies634 entitled “The Present Status of Folic Acid” is available.

The literature is confusing and difficult to evaluate. Many divergent approaches have been used. These approaches have now, in part at least, converged. As further knowledge is gained and made known it can be expected that further simplification will take place.

In this review the various factors will be discussed separately although many of them have been shown to be identical (table 2). This is now a somewhat historical approach and is used with that purpose in mind as well as for clarity. The hematologic manifestations of these factors, or factor, will be discussed separately and by species.

1. Norite eluate factor.—In 1940 Snell and Peterson76 reported that Lactobacillus casei, for maximal growth, required in addition to the basal media a norite filtrate factor and a norite eluate factor, both obtained from yeast. They demonstrated that the activity of the filtrate factor was due to the content of pyridoxine and biotin. Studies on the norite eluate factor were reported. ‘‘Solubilized’’ liver was found to be a rich source. The substance was heat stable, very labile to acids and
reducing agents, somewhat more stable to alkali and oxidizing agents, and resistant to enzyme action. The active substance was precipitated by phosphotungstic acid, basic lead acetate, copper sulfate, and sodium bisulfide. It was easily absorbed by lead sulfide and fuller's earth and was insoluble in all common organic solvents except glacial acetic acid, formamide, and dioxane. Hutchings, Bohonos; and Peterson later prepared a purified concentrate of the eluate factor and presented evidence that the active principle was an acid and contained an amino group. They also found that their factor stimulated the growth of Streptococcus lactis R as well as L. casei. This factor is now considered to be identical with the one to be next described.

2. Lactobacillus casei factor from liver.—Stokstad prepared a concentrate of a factor in liver which had microbiological activity for L. casei. He reported that the substance contained nitrogen, phosphorus, a pentose and guanine and concluded that the substance was a nucleotide. He found that the factor was partially replaceable by thymine and guanine. Later work with purer concentrates showed it to be free of phosphorus. This compound has now been isolated and synthesized. Both the natural and synthetic compounds are equally active when assayed by L. casei or S. lactis R. The formula has recently been announced and is as follows:

![Chemical structure of the L. casei factor](image)

\[N-\{4-\{3-\text{amino}-4\text{-hydroxy}-6\text{-pteridyl}\text{ methylaminobenzoyl glutamic acid}\}]\]
Pteroylglutamic acid is the chemical name which has been suggested for this compound. The synthetic substance has been placed on the market under the name “folic acid.”

3. Folic acid.—Mitchell, Snell, and Williams\cite{82} prepared a concentrate from spinach which stimulated the growth of *Streptococcus lactis R*. This compound was 100 times more active than Stokstad’s original preparation. From diffusion experiments the compound appeared to have a molecular weight of about 500. Later the molecular weight was reported to be more nearly 400.\cite{83} Their preparation was essentially a pure compound and because it was obtained from a leafy material was named folic acid. The active material has now been obtained from liver, yeast, milk, casein, peptone, and other natural sources. Williams and his group\cite{88,86} have obtained highly purified concentrates which are 137,000 times more active than a standard liver extract. They have reported in detail their concentration methods as well as absorption studies and chemical and physiological properties of the active material. The compound is markedly unstable to oxidation, reduction, acid, alkali, light, dry heat, acetylation, esterification, methylation, benzolation, nitrous acid, bromine, and hypobromite. It is highly soluble in water, glacial acetic acid, and liquid ammonia but is essentially insoluble in methanol, ethanol, butanol, acetone, ether, dioxine, benzene, petroleum ether, and chloroform. The ammonium salt is quite soluble in aqueous alcohol and is very soluble in water. They have indicated that the formula may be approximately $C_{15}H_{20}O_{8}N_{5}$. Absorption studies seem to indicate that folic acid may be converted by various treatments to substances which are very closely related chemically but which have greatly reduced physiological properties. Minor changes in structure seem to produce great changes in the activity for various organisms. This fact may be of significance in considering the next three factors to be described.

Thymine replaces folic acid if given in sufficient quantities.\cite{78,87,88} Approximately 5,000 times more thymine is required than folic acid and although thymine can completely replace folic acid in the nutrition of *S. lactis R* it does not completely replace folic acid activity in the nutrition of *L. casei*. Stokes\cite{87} has suggested that folic acid participates directly or indirectly as a coenzyme in the synthesis of thymine or a related compound in the lactic acid streptococci. Folic acid has not been detected in streptococci grown in thymine medium. Hypoxanthine, alloxazine, alloxantine, guanidine, theobromine, xanthopterin, alloxan, allantoin, and uric acid have slight activity at high concentrations. Absorption studies indicate that folic acid contains in its structure a unit very similar to xanthopterin.

Originally folic acid was considered to be a different substance from the norite eluate factor and the liver *Lactobacillus casei* factor of Stokstad because the compound contained no phosphorus and was active for *Streptococcus lactis R*. These two differences were later reconciled when Stokstad\cite{79} and Hutchings et al.\cite{77} demonstrated that their factors did not contain phosphorus and when it was demonstrated that the norite eluate factor stimulated the growth of *Streptococcus lactis R*\cite{77} and that folic acid was equally effective for *L. casei*.\cite{82,89,90} However, since the folic acid preparation was not entirely pure it is possible that it contained several of the factors to be mentioned in addition to folic acid. Hutchings, Stokstad,
Bohonos, and Slobodkin have presented evidence on the basis of absorption spectra that folic acid is different from the liver compound which they isolated.

4. *Streptococcus lactis R* factor of Kefesztesy, Riches, and Stokes.—These workers isolated a growth factor "from various types of extracts and liver preparations" which they believed was neither folic acid nor the norite eluate factor. The substance was found to be 1500 times more active for *Streptococcus lactis R* than for *Lactobacillus casei*. Sebrell noted that this factor was inactive in alleviating the anemia and leukopenia occurring in rats following the administration of sulfaguanidine in addition to a purified diet.

Using the same methods of synthesis as for pteroylglutamic acid except for the substitution of p-aminobenzoic acid for p-aminobenzoyl-1 (+)-glutamic acid, a compound, pteroic acid, has been obtained which is active for *Streptococcus lactis R* but inactive for *Lactobacillus casei* and the chick. It is possible that this factor is identical with Kefesztesy’s factor.

5. Yeast factor of Stokstad.—Stokstad isolated a compound from yeast which on the basis of microbiological assay he believed to be distinct from the liver *L. casei* factor. The preparation from yeast was found to be only half as active for *Streptococcus lactis R* as for *L. casei*.

6. Factor of Hutchings et al.—Hutchings, Stokstad, Bohonos, and Slobodkin isolated a factor in crystalline form from an unstated source which was active for *L. casei* and *S. lactis R* and also active in the nutrition of the chick but which they believed on the basis of absorption spectra to be different from folic acid and the *L. casei* factor isolated from liver. This new compound was 85 to 90 per cent as active as that from liver when assayed with *L. casei*, but only 6 per cent as active as the liver compound by *S. lactis R* assay. This substance is known as the "fermentation compound" and has been found to differ from pteroylglutamic acid in that it contains two additional glutamic acid groups. Day et al. found that treatment of this preparation with the enzyme solution of Mims et al. greatly increased its activity toward *S. lactis R*.

7. Vitamin M.—In 1935 Day, Langston, and Shukers reported that young monkeys maintained on a diet of casein, whole wheat, polished rice, cod liver oil, orange, and a salt mixture supplement lost weight, developed diarrhea and gingivitis, and died between the 26th and 100th day of a fulminating fatal blood disorder characterized by anemia, leukopenia, and thrombocytopenia. Likewise, monkeys maintained on a modified Goldberger black-tongue-producing diet consisting of corn, cowpeas, casein, cottonseed oil, cod liver oil, and salt mixture developed a similar syndrome. These observations have been amply confirmed by other investigators. The addition of the diet of ascorbic acid, nicotinic acid, riboflavin, thiamine, copper, iron, various types of concentrated liver extract including "anahaemin," pantothenic acid, pyridoxine, choline, pimetic acid, glutamine, inositol, p-aminobenzoic acid, banana, and heated liver extract failed to prevent the nutritional cytopenia. The deficient diet supplemented with either dried brewer’s yeast or two grams of liver extract (Cohn fraction G), daily, supported good growth, promoted normal body development, and maintained a normal blood picture over long periods of time. When liver
extract was added to the diet of a deficient animal with profound anemia and leukopenia a dramatic reticulocytosis occurred and was followed by ultimate recovery. The authors proposed the term "vitamin M" for the factor which prevented the nutritional cytopenia in the monkey. Wilson, Doan, Saslaw, and Schwab reported that the addition of folic acid concentrate to the diet restored normal white cell equilibrium. Totter and co-workers assayed a number of substances for folic acid by the S. lactis R method and were unable to find a correlation between folic acid and vitamin M content. However, materials which contained very little folic acid but which were good sources of vitamin M were found to give an increase in S. lactis R assay when incubated with an enzyme preparation from rat liver. Following such an enzymatic conversion of the potential S. lactis R-stimulating factor there was excellent correlation with the vitamin M activity. They then suggested that vitamin M, vitamin B conjugate, the potential S. lactis R-stimulating factor and the factor antagonistic to the succinylsulfathiazole effect in rats were probably similar if not identical. They have now demonstrated that vitamin M-deficient monkeys respond to the crystalline L. casei factor of Hutchings et al.

Xanthopterin.—Xanthopterin, the yellow pterin pigment of butterfly wings, was isolated from the wings of the Colias philodice in 1936 by Schöpf and Becker. The compound was synthesized by Purrmann in 1940 and recently a convenient method for its synthesis has been reported by Totter. The compound has been isolated from human urine and liver. Urinary xanthopterin is known as uropo-terin.

Sir Frederick Gowland Hopkins first suggested that pterins play a role in hemopoiesis. In 1936 Tschöche and Wolf reported that pterins and particularly xanthopterin have hemopoietic activity when administered to rats made anemic on a diet of goat's milk. Simmons and Norris reported in 1941 that the anemia occurring in Chinook salmon maintained on a high protein diet in which the vitamin B complex was supplied in the form of yeast responded to injections of 50 γ of crystalline xanthopterin. This has since been confirmed. The photoisomer of xanthopterin was found to be lethal in a similar dose.

Evidence that xanthopterin is closely related to folic acid has been supplied. Wright and Welch in experiments with surviving rat livers have shown that, following the incubation of rat liver with xanthopterin, more folic acid is found on microbiological assay of the digestion mixture than is present in a similar amount of rat liver alone. They also found a substance in urine which was stable to both heat and acid and which occurred in both a free and combined form. When the substance was incubated with fresh rat's liver there was a demonstrable increase in the folic acid content. The evidence suggested that this compound is related to xanthopterin. Williams and his group, from absorption studies of xanthopterin and folic acid, have suggested that folic acid contains a structural unit very similar to xanthopterin. Bloom et al. state that the ultraviolet absorption characteristics of vitamin B complex coupled with the nitrogen content of the compound suggest the presence in the molecule of a pyrimidopyrazine ring structure such as a pterin. Totter and co-workers found that the addition of
2.5 to 10 mg. of synthetic xanthopterin to vitamin M-deficient monkeys resulted in a reticulocytosis in 3 to 6 days which lasted 2 to 5 days. White and red cell counts increased to normal in 3 to 13 days and remained normal for varying periods. Synthetic xanthopterin alone when given to prevent cytopenia failed to protect completely but did delay the onset of nutritional cytopenia. In one animal given heated liver (previously shown to be ineffective by itself) plus xanthopterin, the white and red cell counts were still normal after 71 days. Cessation of xanthopterin therapy resulted in a prompt return of the cytopenia and resumption of this therapy resulted in a response similar to the first. They also noted that the livers obtained from vitamin M-deficient monkeys were low in preformed folic acid and that when the livers were incubated with xanthopterin and yeast or yeast alone there was a rise in the folic acid content. These authors concluded that the monkey requires xanthopterin as well as unidentified factors in liver for hemopoiesis.

Totter and Day reported that in rats xanthopterin was effective in alleviating the leukopenia and countering the growth inhibition produced by adding 1 per cent succinylsulfathiazole to a synthetic diet complete in all known members of the vitamin B complex. Others have been unable to confirm this and the authors now state that they are unable to repeat their own work. O'Dell and Hogan have stated that xanthopterin is inactive when fed to nutritionally anemic chicks, and Totter, Mims, and Day have found that chicken liver fails to produce any extra folic acid from xanthopterin alone.

9. Vitamin B.-In 1940 Hogan and Parrott presented evidence for the existence of an unidentified factor necessary in addition to the known vitamins for the prevention of anemia in chicks. A factor in liver designated vitamin B was found to prevent the development of the anemia. This anemia has now been characterized and studied, and the preventive factor isolated in crystalline form. All the available evidence indicates that the substance is identical with the L. casei factor isolated from liver and the norite eluate factor and is at least similar to folic acid.

O'Dell and Hogan in 1943 pointed out that vitamin B was acidic in nature, formed salts with heavy metals, was destroyed by mineral acids, stable to alkali, adsorbed on fuller's earth and norite, eluted by ammonia, destroyed by oxidation, and insoluble in the common organic solvents. Analysis of an ash-free specimen gave the following percentage composition: C 52.44, 52.46; H 4.28, 4.49; N 19.8, 19.6. The ultraviolet absorption characteristics coupled with the nitrogen content suggest the presence in the molecule of a pyrimidopyrazine ring structure such as a pterin. A comparison of the ultraviolet absorption curves for xanthopterin and vitamin B shows very striking dissimilarities although in general they are quite similar. The fact that synthetic L. casei factor is effective in preventing the anemia in chicks, that crystalline vitamin B is a highly active growth factor for L. casei and is effective in correcting the anemia in rats maintained on a purified diet supplemented with sulfone compounds together with the demonstration that the vitamin B potency of the livers from rats receiving succinylsulfathiazole is markedly reduced is good evidence that the L. casei liver factor and vitamin B are identical. Furthermore, both factors have similar solubilities and both are adsorbed on fuller's earth at acid pH levels.
Vitamin B<sub>c</sub> conjugate.—Binkley et al.<sup>138</sup> observed that certain yeast extracts were highly active in vitamin B<sub>c</sub> activity as measured in the anemic chick but they had little potency in stimulating the growth of L. <i>casei</i>. Only about 2 to 5 per cent of the chick antianemic activity could be accounted for in terms of microbiological growth effect on either <i>L. casei</i> or <i>S. lactis</i> <i>R</i>. The methods used for isolating vitamin B<sub>c</sub> failed when applied to yeast. They noted, however, that the concentrates of the chick antianemic factor from yeast which were essentially inert in stimulating growth of <i>L. casei</i> became highly active in microbiological growth effect following enzymatic digestion. Procedures used for the isolation of vitamin B<sub>c</sub> when applied to such digests yielded a pure crystalline compound which had the same growth-stimulating activity on <i>L. casei</i> and <i>S. lactis</i> <i>R</i> as vitamin B<sub>c</sub> from liver and also produced a comparable effect on the blood picture and growth of chicks. The products from the two sources also had the same color, crystalline appearance, solubilities, charring points, crystallographic pattern, ultraviolet absorption spectra, and elementary chemical analysis. They referred to the chick antianemic factor in yeast as vitamin B<sub>c</sub> conjugate and the enzyme which formed vitamin B<sub>c</sub> from it, vitamin B<sub>c</sub> conjugase.<sup>139</sup> Subsequent work resulted in the isolation of vitamin B<sub>c</sub> conjugate.<sup>140</sup> The pure crystalline substance has an elementary composition of C 49.61 per cent; H 5.36 per cent; N 14.79 per cent. Comparison of the specific ultraviolet absorption properties of this compound with vitamin B<sub>c</sub> reveals that both have the same chromophoric groups. It appears that the molecular size of vitamin B<sub>c</sub> conjugate is 2.8 times that of vitamin B<sub>c</sub>. The data demonstrate that the vitamin B<sub>c</sub> present in the conjugate molecule as calculated from the ultraviolet absorption data and as found by enzymatic microbiological assay is available to the chick. The new crystalline product is only slightly stimulating to the growth of <i>L. casei</i> and <i>S. lactis</i> <i>R</i> and can be clearly differentiated from the various <i>L. casei</i> factors on this basis as well as by the ultraviolet absorption constants. This substance is now known to be pteroyl-heptaglutamic acid.<sup>141</sup> Vitamin B<sub>c</sub> conjugase has been found to be widely distributed in nature.<sup>140, 111</sup> Hog kidney, liver, small intestine, and beef liver are rich sources. It occurs in sweet almond and to a lesser extent in potatoes. Only traces have been found in molds and none in yeast.

Vitamins B<sub>10</sub> and B<sub>11</sub>.—Briggs et al.<sup>112-115</sup> have demonstrated the existence of two water-soluble vitamins which they believe to be separate from the various factors just described and which are needed by the chick for maintenance of normal hemoglobin values, proper feather formation, and normal growth. The factor essential for feather formation has been named B<sub>10</sub> and the factor necessary for growth B<sub>11</sub>. Both factors are effective in preventing the development of anemia. The anemia is stated to be macrocytic and accompanied by leukopenia. From their studies they conclude that both B<sub>10</sub> and B<sub>11</sub> "although distinct entities, seem to be related chemically to the various factors with vitamin B<sub>c</sub> activity, since the properties are so similar and because compounds with vitamin B<sub>c</sub> activity have some vitamin B<sub>10</sub> and B<sub>11</sub> activity when fed alone to the chick."

It has now been demonstrated that the addition of 25 γ of synthetic folic acid...
GEORGE E. CARTWRIGHT

(pteroylglutamic acid) per 100 grams of basal ration entirely prevents the reduced growth, poor feathering condition, and low hemoglobin and hematocrit values consistently obtained when the basal ration is fed to chicks.6455 Thus all vitamin B10 and B11 activity is due to folic acid.

Briggs et al.145 have suggested the existence of another unknown factor necessary to maintain normal hemoglobin formation in the chick in addition to those described above.

12. Pyracin.—Scott et al.146, 147 have presented evidence that either the lactone of 2-methyl-3 hydroxy-4 carboxy-5 hydroxymethylpyridine (γ-pyracin) is required in addition to the Lactobacillus casei factor for the complete prevention of the macrocytic anemia that develops in chicks fed a purified diet. The results of hematological studies showed that when the L. casei factor alone was added to the diet, normocytic, hypochromic anemia developed. When β-pyracin was added alone, a macrocytic, normochromic anemia developed. Furthermore, they demonstrated that when pure L. casei factor and pyracin were incubated with liver there was a marked increase in the S. lactis R-stimulating potency.148 They have suggested that pyracin may form a conjugate with the L. casei factor or that it may enter into an enzyme system which brings about the conversion. These results have not been confirmed by others and Hutchings, Oleson, and Stokstad144 have found that the addition of β-pyracin is not necessary for growth or hemoglobin formation in addition to the synthetic liver Lactobacillus casei factor.

13. Hematological manifestations of the deficiency.—a. Rat.—Rats fed sulfonamides at a level of 1 to 2 per cent in purified diets develop a severe granulocytopenia, leukopenia, thrombocytopenia, and normocytic anemia in 4 to 6 weeks. The white cell count falls from a level of 10 to 16 thousand down to 1 to 4 thousand. Blood smears are reported as showing marked abnormalities in the size, shape, and staining reactions of the red cells and an increase in the number of nucleated forms occurs. The anemia has been reported by some authors as being hypochromic. The granulocytopenia is pronounced and there may also be a reduction in the absolute number of lymphocytes. Severe anemia may be produced regularly in rats fed a sulfasuxidine-containing purified diet and subjected to hemorrhages, whereas rats fed a purified diet alone do not develop anemia when bled to the same extent. Granulocytopenia has been observed in a small percentage of rats fed purified diets without sulfonamides.

The histologic picture of the bone marrow varies from almost complete aplasia to intense hyperplasia. The total nucleated cell count is only slightly less than normal. However, the majority of marrow cells are undifferentiated primitive forms with relative increases in myeloblasts and early erythroblasts. Myelocytes, adult granulocytes, late erythroblasts and megakaryocytes practically disappear. Thus there is a maturation arrest in the early stages of the development of the three cellular elements of the blood.

Crystalline L. casei factor has been found to have a preventive as well as a corrective action on the anemia, leukopenia, and thrombocytopenia. A concentrate of vitamin B1 has also been shown to be effective.

Rats fed thiouracil in a purified diet develop anemia and, in lesser incidence,
leukopenia. Animals which receive, concomitantly, thyroxin injections or thyroid powder become granulocytopenic and leukopenic. The granulocytopenia and leukopenia of these rats may be corrected by treatment with synthetic \textit{L. casei} factor.\textsuperscript{642}

\textit{b. Chick.} — The characteristics of the anemia, the leukopenia, and the thrombocytopenia developing in chicks on purified rations deficient in vitamin \textit{B}\textsubscript{12} are summarized in table 3. All workers are in agreement that the anemia is macrocytic\textsuperscript{131, 147, 169-162} and that the mean corpuscular hemoglobin is increased. There is no agreement as to the mean corpuscular hemoglobin concentration. Various workers have found that the anemia is hypochromic,\textsuperscript{160, 162} normochromic,\textsuperscript{147} or hyperchromic.\textsuperscript{131, 162}

Anemia is detectable after 7 days. Thereafter it develops rapidly and after 18 days on the deficient ration the anemia is pronounced. After about 9 days the immature red cells are increased and polychromatophilia and basophilia are evident. Macrocyaetes with a great variety of sizes and shapes appear on about the 14th day. By the 21st day numerous normoblasts, pronormoblasts, and myeloblasts are evident. Active mitotic figures are common. At 28 days the nucleated red blood cells present a moderate to marked anisocytosis and poikilocytosis. Polychromasia, basophilia, and macrocytosis are extremely marked. In addition the nuclei of many of the immature cells become eccentrically placed and the nuclear chromatin breaks into fragments within the cells.

The pattern of the leukocytes deviates considerably from the normal. After about 9 days the distribution, size, number, and staining reactions of all white cells become progressively variable as leukopenia develops. The neutrophils appear pyknotic, the nuclei become hypersegmented and acquire a lighter staining characteristic. Vacuoles appear in the cytoplasm of the lymphocytes after the 14th day. By the 28th day the leukopenia is pronounced. The absolute number of neutrophils is maintained. Lymphopenia is extreme and there is also a reduction in the number of eosinophils, basophils, and monocytes.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>\textit{B}, Def.</th>
<th>\textit{B}, Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. B. C. millions cu. mm.</td>
<td>2.27</td>
<td>0.93</td>
<td>2.12</td>
</tr>
<tr>
<td>Hemoglobin Gm. cc.</td>
<td>7.74</td>
<td>4.76</td>
<td>8.05</td>
</tr>
<tr>
<td>Volume packed R. B. C. cc.</td>
<td>31.2</td>
<td>15.0</td>
<td>30.7</td>
</tr>
<tr>
<td>Mean Corpuscular Volume cu. u.</td>
<td>137</td>
<td>161</td>
<td>145</td>
</tr>
<tr>
<td>Mean Corpuscular Hemoglobin</td>
<td>34</td>
<td>31</td>
<td>38</td>
</tr>
<tr>
<td>Mean Corpuscular Hemoglobin conc. %</td>
<td>2.5</td>
<td>32</td>
<td>26</td>
</tr>
<tr>
<td>W. B. C. per cu. mm.</td>
<td>29.935</td>
<td>7.690</td>
<td>24.440</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>5.733</td>
<td>5.395</td>
<td>5.965</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>449</td>
<td>274</td>
<td>244</td>
</tr>
<tr>
<td>Basophils</td>
<td>659</td>
<td>197</td>
<td>415</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>22.406</td>
<td>1.756</td>
<td>16.961</td>
</tr>
<tr>
<td>Monocytes</td>
<td>688</td>
<td>68</td>
<td>855</td>
</tr>
<tr>
<td>Thrombocytes cu. mm.</td>
<td>31.180</td>
<td>18.020</td>
<td>32.620</td>
</tr>
</tbody>
</table>

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The thrombocytes undergo considerable alteration in size, shape, and numbers. Karyorrhexis and pyknosis develop. After 3 to 4 weeks there is an increase in the vacuolation of the cytoplasm along with a general swelling of the granules. The degree of thrombocytopenia is extremely variable but generally is not extreme.

The addition of 5 to 10 micrograms of pure crystalline vitamin B₁₂ per 100 grams of basal ration prevents the appearance of severe anemia. The addition of 20 to 40 micrograms per 100 grams of basal ration gives nearly optimal protection against anemia and thrombocytopenia, whereas 20 to 200 micrograms is sufficient for the maintenance of normal hemopoiesis in 4 weeks old growing chicks. Synthetic L. casei factor from liver has also been shown to be effective in preventing the anemia.

c. Monkey.—Monkeys maintained on a diet of refined foodstuffs (vitamin M deficiency) or on a purified diet supplemented with nine crystalline members of the vitamin B complex plus ascorbic acid but deficient in the L. casei factor and related substances develop anemia, leukopenia, and thrombocytopenia. The anemia is reported to be normocytic. The most characteristic feature of the syndrome is the leukopenia. The total white count is usually reduced from a normal value of 15,000 to 2,000-3,000 per cu. mm. A few animals have developed white counts of less than 1,000 per cu. mm. All of the white blood cell types are involved although there is considerable variation from animal to animal in regard to cell type distribution. However, there is almost invariably an absolute neutropenia as well as an absolute lymphopenia.

The peripheral blood smears have not been studied carefully from the standpoint of red blood cell morphology. It has been stated that the bone marrow of animals dying from severe leukopenia shows a relative and absolute hypoplasia of the myeloid elements but detailed studies of the bone marrow have not been reported.

d. Human subjects.—The value of folic acid (pteroylglutamic acid) in various types of macrocytic anemia in relapse has now been adequately demonstrated and repeatedly confirmed.

Spies et al. observed a reticulocyte response and an increase in the red cell count in patients with macrocytic anemia in relapse. The drug was given orally to 4 patients in a dose of 100 to 150 mg. daily and parenterally to 5 patients in a dose of 20 to 50 mg. It was not stated whether these patients had nutritional macrocytic anemia or Addisonian pernicious anemia.

Moore, Bierbaum, Welch, and Wright noted clinical and hematologic remissions in 2 patients with Addisonian pernicious anemia following the daily oral administration for 10 days of 30 mg. and 100 mg. of the synthetic material, respectively. One of the patients had an initial red count of 1.2 million cells per cu. mm. This patient developed a reticulocyte peak of 40 per cent on the 7th day of therapy. The other patient had an initial red blood cell count of only 0.7 to 0.95 million. In this patient a maximal reticulocytosis of 44.5 per cent was reached on the 8th day. Leukopenia and thrombocytopenia were also present and were corrected by the administration of L. casei factor. The response of this patient is shown in figure 1.
Observations on the effectiveness of synthetic folic acid in the treatment of Addisonian pernicious anemia have now been confirmed many times. The first change noted is an improvement in the general well-being of the patient on about the 3rd day of therapy. This is followed in a day or so by a marked reticulocytosis reaching a maximum between the 6th and 10th days of therapy. There is a gradual rise in leukocytes, platelets, red cells, hemoglobin, and volume of packed red cells to normal. The immediate response is quantitatively and qualitatively equal to that following liver extract therapy. The effect of synthetic folic acid on the neurological manifestations of pernicious anemia remains to be determined. The exact minimal and optimal doses of folic acid have not been determined but a daily dose of 5 to 10 mg. either orally or parenterally will often produce a maximal response.

Synthetic folic acid has been found to be effective in alleviating the hematologic as well as many of the other manifestations of sprue. This observation was first made by Darby and Jones and has since been confirmed by them as well as by others. The substance is effective in both nontropical and tropical sprue. The administration of 15 to 200 mg. daily of the drug is followed by a reticulocytosis and rise in the leukocytes, platelets, red count, hemoglobin, and volume of packed red cells to normal. The glossitis disappears in 3 to 4 days, after which there is rapid regeneration of papillae. There is an improved sense of well-being, an improvement in appetite, and a decided gain in body weight. The sternal marrow returns to normal. Improvement in the absorption of glucose and water-soluble vitamins has been noted. Spies and his associates in Cuba and Puerto Rico have observed a profound effect on the alimentary tract of persons with sprue and state: "Roentgenograms of the gastro-intestinal tract showed that the highly irritated bowel tends to become normal. There is a marked improvement in the diarrhea. The number of stools per day decreases and the feces, loose and bulky before therapy, usually become formed and essentially normal in appearance."

Darby, Jones, and Johnson failed to observe improvement in the absorption of fat in 2 cases during a 2 month observation period. Spies likewise has found that the steatorrhea of the more chronic cases is little affected.

Synthetic folic acid has been found to be effective in the treatment of nontropical nutritional macrocytic anemia, macrocytic anemia of infancy with megaloblastic bone marrow, macrocytic anemia of pregnancy, macrocytic anemia associated with alcoholic cirrhosis of the liver, and macrocytic anemia associated with carcinoma of the stomach.

Watson, Sebrell, McKelvey, and Daft noted an increase in the leukocyte count after the daily oral administration of 5 mg. of L. casei factor to 7 patients who had developed leukopenia following roentgen-ray therapy. Berry, Spies, and Doan administered synthetic folic acid parenterally to leukopenic malnourished patients and noted a transient rise in the leukocytes in some cases.

No response to folic acid has been observed in cases of aplastic anemia, leukemia, or iron deficiency anemia. Folic acid has not been found to be of value in correcting the leukopenia of influenza, or the panhematopenia of myelophthisic and idiopathic states. Newman and Jones noted the failure of crystalline
folic acid to prevent the development of agranulocytosis in a patient receiving thiouracil.

Spies and co-workers\textsuperscript{659-661} have administered large doses (4 to 12 grams) of thymine (5-methyl uracil) to 10 cases of Addisonian pernicious anemia. The clinical and hematological improvement was found to be similar to that which follows the administration of folic acid to such patients. The exact mode of action of thymine is obscure but the authors speculate that perhaps folic acid acts as an enzyme or coenzyme in the synthesis of thymine or a thymine-like compound.

The recent advances in the therapy of Addisonian pernicious anemia just described lead naturally to questions as to the identity of the extrinsic factor, the intrinsic factor, and the antipernicious anemia substance in liver as postulated by Castle. It can be stated that the folic acid is probably not the extrinsic factor alone since it acts orally in the absence of normal human gastric juice and is effective parenterally. However, as Moore\textsuperscript{168} points out, caution must be used in making this differentiation since there is no information as to what effect Castle's extrinsic factor would have were it to be injected intravenously in pure form. That folic acid is identical with the effective substance in liver is unlikely for two reasons. At least 2 to 5 mg. daily are necessary to obtain an effective response. Good responses with as little as 0.7 mg. of highly purified liver extract given intravenously for 3 days have been obtained.\textsuperscript{169} Secondly, purified commercial liver extract does not possess significant \textit{L. casei activity} to explain its therapeutic activity.\textsuperscript{185, 170} Welch, Heinle, Nelson, and Nelson\textsuperscript{662} have recently made several interesting observations. They found that pteroylglutamic acid (folic acid) was released from pteroylheptaglutamate (vitamin B\textsubscript{12} conjugate) in a normal individual but not in a patient with pernicious anemia. When 2 pernicious anemia patients were given injections of purified liver extract the urinary excretion of pteroylglutamic acid (\textit{L. casei factor}) doubled. The addition of purified liver extract to sternal marrow from patients both with and without pernicious anemia frequently augmented the formation of \textit{L. casei factor} from added pteroylheptaglutamate (\textit{in vitro} at pH 4.5 but not at pH 7.0). Surprisingly, however, when pteroylheptaglutamic acid was incubated with normal human gastric juice no free folic acid was released and when a patient with pernicious anemia in relapse was given the heptaglutamate orally for 11 days with normal human gastric juice no reticulocyte response occurred. This latter finding is difficult to explain but in general the results seem to indicate that in pernicious anemia it is the conjugase system which is at fault and that a constituent of liver extract may be a component of the conjugase system or may counteract the effect of conjugase-inhibiting substances. These findings would account, in part at least, for the failure of pernicious anemia patients to derive adequate amounts of folic acid from the diet since in nature the compound may occur principally in the conjugated form.

E. Extrinsic Factor.—It has now been 17 years since Castle first presented evidence concerning a dietary factor which when incubated with a factor in normal human gastric juice formed a substance effective in the treatment of pernicious anemia.\textsuperscript{172-175} As each new B vitamin has appeared it has been tested in the hope that it might be the active factor. It has now been demonstrated that thiamine,
nicotinic acid, pyridoxine, pantothenic acid, p-aminobenzoic acid, choline, inositol, biotin, and xanthopterin are all inactive given either singly or in combination. The possible identity or relationship of the extrinsic factor with one of the substances of the L. casei factor group, especially pteroylheptaglutamic acid, has just been discussed.

Beef muscle, yeast, wheat germ, milk, purified casein, liver, eggs, and rice polishings have been shown to contain significant amounts of the factor. Several commercial "vitamin-free" casein preparations contain extrinsic factor according to Castle. Complete removal can be attained by repeated precipitation or by extraction with dilute acid or with alcohol. The active factor can be removed from beef muscles by repeated extractions with dilute acetic acid or by extraction with 70 to 80 per cent alcohol. It is water soluble, thermostable, resistant to alkalization, readily soluble in 70 to 80 per cent alcohol but rapidly destroyed in 95 per cent alcohol. The active substance is ultrafiltrable and cannot be extracted from an 80 per cent alcoholic solution with ether. Following saturation of an alcoholic extract of beef muscle with ammonium sulfate the activity is found in the precipitate.

The existence of an anemia due to a deficiency of Castle’s extrinsic factor has been clearly demonstrated by Moore, Vilter, Minnich, and Spies. These workers have described the occurrence of macrocytic anemia in 56 patients who had existed for years on diets inadequate in animal protein and in the vitamin B complex. Most of these subjects had at one time shown clinical evidence of pellagra, arboflavinosis, or beriberi. The most striking clinical manifestations were weakness, pallor, glossitis, skin changes; and intermittent or persistent diarrhea. Eighteen of the patients showed signs of mild peripheral neuritis, but combined system disease was not observed. The anemia as well as the bone marrow was cytologically indistinguishable from true Addisonian pernicious anemia. The mean corpuscular volume ranged from 105 to 163 cubic microns and the mean corpuscular hemoglobin concentration from 27 to 38 per cent. Blood smears showed marked anisocytosis, poikilocytosis, polychromatophilia, and an occasional nucleated red cell. In most cases there was an associated neutropenia and thrombocytopenia. Nuclear hypersegmentation of both neutrophils and eosinophils was common. The bone marrow showed a shift to the younger forms of nucleated erythroid cells with many megaloblasts and early erythroblasts. Free hydrochloric acid was found in the gastric contents of 18 patients. In 5 of these the acid was present only intermittently. In 4 patients a persistent achlorhydria was demonstrated. All evidences of increased hemolysis were absent and the serum iron values were either low or in the normal range. Following the administration of an 80 per cent alcoholic extract of beef muscle a reticulocytosis developed and after prolonged administration the erythrocytes increased in number. All of the subjects showed a prompt therapeutic response to the parenteral injection of highly purified liver extracts. Studies on the pathogenesis of the anemia indicated that it was probably caused by a dietary deficiency of extrinsic factor associated in many, but not all, instances with poor absorption from the intestinal tract. Inadequate production of intrinsic factor was thought to be a complicating factor in some of the cases. Thiamine,
nicotinic acid, riboflavin, calcium pantothenate, pyridoxine, inositol, para-aminobenzoic acid and choline given together both orally and parenterally were ineffective in relieving the anemia. The anemia, leukopenia and thrombocytopenia all respond rapidly to pteroylglutamic (folic) acid therapy. Wills reported the occurrence of macrocytic anemia of nutritional origin in India. The anemia is present in the lower classes which subsist on a low protein diet consisting principally of cereal grains. Although the blood and bone marrow pictures are indistinguishable from pernicious anemia there are several important differences. Nutritional macrocytic anemia is not accompanied by achlorhydria, the disease has an earlier age incidence, although it occurs equally in the two sexes, the anemia is frequently associated with pregnancy, there is no evidence of increased blood destruction, there is an absence of neurological involvement, and the anemia fails to respond to the purified liver extract anahaemin which is so effective in the treatment of Addisonian pernicious anemia. The condition responds promptly to crude liver extracts such as campolon or to marmite, an autolysed yeast extract. On the basis of the response to marmite, a substance which is ineffective in true Addisonian anemia, and its failure to respond to anahaemin Wills concluded that the anemia is due to a deficiency in the diet of some factor at present unidentified but other than Castle's extrinsic factor. Napier, after studying nutritional macrocytic anemia in Calcutta, arrived at essentially the same conclusions except that, in addition, he found a group in which 'anahaemin' was effective; in these he felt that malaria was a predisposing factor. Foy and Kondi have reported the occurrence of a nutritional macrocytic anemia in Macedonia similar to that reported by Wills but differing in two important respects: the anemia was associated with a high indirect van den Bergh reaction and responded rapidly to 'anahaemin.' In a detailed description of nutritional macrocytic anemia in Macedonia Fairley, Bromfield, Foy, and Kondi distinguished two distinct subgroups, hemolytic and nonhemolytic. Many of their cases were complicated, however, by malaria, splenomegaly and parasitic infestation, and leukopenia and purpuric manifestations were often present. They concluded that the nonhemolytic cases were due to an uncomplicated dietary deficiency and suggested that in the hemolytic group chronic malaria supplied an additional hemolytic factor. Their cases responded slowly to large doses of marmite or to 'campolon' injections. Fairley has since reported an instance of this type of anemia in an Indian which responded satisfactorily to 'anahaemin.' Others have recorded a response to cruder preparations. Rodriguez-Molina has reported 2 cases occurring in Puerto Rico. One of these responded to purified liver extract and the other to marmite. Macrocytic anemia in Kenya has been reported by Anderson and Roberts. Whether or not these anemias reported from various parts of the world are due to a deficiency of Castle's extrinsic factor cannot be determined from the confusing and conflicting evidence now available concerning their response to various liver fractions. It would be interesting to know if these tropical nutritional macrocytic anemias respond to the various pteroylglutamic acids.

Recently Watson and Castle have made observations on 3 female patients.
with nutritional macrocytic anemia occurring in the United States. These patients failed to respond to parenteral liver therapy but responded promptly to orally administered liver extract. They concluded that these patients had a deficiency of some substance other than the principle effective in pernicious anemia and that the "unitarian" hypothesis concerning the etiology of pernicious anemia and other nutritional macrocytic anemias did not apply to these patients. It is now known that this type of anemia responds to pteroylglutamic acid. This would be expected since these patients probably have a deficiency of folic acid rather than a faulty conjugase enzyme system as in true pernicious anemia.

Wills99-96 produced a macrocytic anemia with a megaloblastic marrow in monkeys by maintaining them on a diet based on one in common use among poorer class Mohammedans in Bombay, where nutritional macrocytic anemia is common. The diet consisted of polished rice, margarine, salt, iron, white bread, cod liver oil, and either tomatoes or carrots. Anemia of severe degree developed. Megaloblasts were constantly present in the peripheral blood at the height of the anemia and normoblasts were significantly increased. In all cases the van den Bergh tests were negative. The anemia responded rapidly with a marked reticulocytosis following the administration of marmite, or following campolon. "Anahaemin," a purified liver extract extremely potent in the treatment of pernicious anemia, was repeatedly ineffective. The active substance in marmite and campolon has been shown to be precipitated by mercuric acetate197 and to be present in the soluble fraction after fractionation with ammonium sulfate. The insoluble fraction which is highly effective in pernicious anemia was ineffective in the treatment of the monkey anemia. From this Wills has concluded that the substance active against the experimental anemia is separate and distinct from the extrinsic factor of Castle. Both substances, however, have very similar characteristics. Both are water-soluble, soluble in dilute acetic acid, resistant to autoclaving, and soluble in 80 per cent alcohol but rapidly inactivated in 90 per cent alcohol. The relationship between the anemia produced by Wills and vitamin M deficiency is not clear. Morphologically they are reported to be quite different. The effect of folic acid on the monkey anemia of Wills has not been reported.

A macrocytic anemia has been produced in rats199 and dogs201 but in both of these animals the anemia was complicated by Bartonella infection. The anemia which Rhoads and Miller202 produced in swine was not definitely macrocytic as they claimed. Cartwright, Wintrobe, and Humphreys663 maintained a single pig on a diet in which highly purified casein (supposedly lacking in extrinsic factor) was substituted for crude casein and to which 2 per cent sulfasuxidine was added. The animal failed to grow normally and developed partial alopecia and a normocytic anemia. Following treatment with a highly purified antipernicious anemia liver extract growth was resumed and the blood returned to normal.

F. Ascorbic Acid.—It has been conclusively shown that the scorbutic state in guinea pigs as well as in human beings is frequently accompanied by anemia. The nature and etiology of the anemia is obscure. The effect of pure ascorbic acid is in dispute.

1. In animals.—Meyer and McCormick203 in 1928 reported the regular occur-
Dietary factors concerned in erythropoiesis

The frequent occurrence of anemia in experimental scurvy in guinea pigs. They suggested, without presenting much evidence, that the anemia was due to increased blood destruction. Mettier and Chew reported reduction in hemoglobin to 6 to 8 Gm. per cent in guinea pig scurvy. Beginning 3 to 4 days prior to the death of the animals increasing numbers of reticulocytes appeared in the peripheral circulation. Examination of the blood smears revealed slight poikilocytosis, anisocytosis, polychromatophilia, and stippling. The bone marrows were hyperplastic with an increased number of normoblasts, suggesting a maturation arrest at this stage. Following the ingestion of 3 cc. of orange juice daily a reticulocytosis began usually on about the 3d day and reached the peak of production within 5 to 7 days. Following the reticulocyte response, there was an increase in the red blood cell and hemoglobin concentrations and a disappearance of the signs of scurvy. The bone marrows showed active and complete red cell maturation. Aron maintained guinea pigs on a scorbutigenic diet supplemented with ascorbic acid for more than 50 days and no anemia developed. When the ascorbic acid supplement was withdrawn anemia developed. The addition of iron failed to prevent the development of anemia when the ascorbic acid was withdrawn. The anemia was quickly relieved in the less severe cases by the administration of ascorbic acid. In other instances ascorbic acid failed and germinated oats were required to relieve the anemia. Sigal has demonstrated that the addition of ascorbic acid to a scorbutigenic diet prevents the development of anemia in the guinea pig. The morphological characteristics of this experimental anemia have not been carefully studied. Edel has reported that both the peripheral blood and the bone marrow show marked erythroblastosis.

2. In human subjects. The frequent occurrence of anemia in human scurvy has been reported by many authors. Mettier et al. state that in a large group of cases of scurvy in adults one may expect to find about one-third with red cell counts between 2 and 3 million per cubic millimeter, about one-third with red cell counts between 3 and 4 million, and the remainder with slight or no anemia. The red cells have been reported as being macrocytic, normocytic, and microcytic or hypochromic. McMillan and Inglis in a study of 40 anemic scorbutic patients found macrocytic anemia in 2, normocytic anemia in 18, simple microcytic anemia in 14, and in 6 patients the anemia was microcytic hypochromic. Nucleated red blood cells were never seen and there was no reticulocytosis. The sternal marrows of 6 patients were examined. Five were normoblastic, 1 megaloblastic. Two of the former showed a few megaloblasts. The presence of megaloblasts was associated with achlorhydria in 2 instances and a low free HCl in another. One patient with a marked macrocytic anemia and normoblastic marrow had normal gastric secretion. As a result of various combinations of therapy they concluded that (1) the anemia observed was of nutritional origin, (2) ascorbic acid alone was not the cause of the anemia, (3) iron deficiency played a very minor part, (4) hemoglobin and red cell regeneration with reticulocytosis occurred in scorbutic patients on a vitamin C-free diet, and finally (5) that ascorbic acid was necessary in some deficient individuals before the anemia would respond to treatment. Parsons stated that in a series of 14 children suffering from scurvy 7 were anemic. The anemia was normocytic
normochromic in 3, normocytic hypochromic in 2, and macrocytic in 1. All of these patients failed to respond to iron but responded to the administration of ascorbic acid without any other alteration in the diet. Vilter and Woolford studied 10 male adults with scurvy, 8 of whom had anemia. The red blood cell counts ranged from 1.74 to 3 million and the hemoglobin levels from 5.8 to 10.5 Gm. The cells were normocytic or moderately macrocytic. The reticulocytes fluctuated between 3 and 10 per cent and the icterus indices ranged from 10 to 22. The urine contained excess urobilinogen but no bile and the blood serum gave the indirect van den Bergh reaction in each instance. The bone marrows were of varying degrees of cellularity and in each case there was an increase in normoblasts. The anemia in all cases responded specifically to ascorbic acid. During the treatment period the patients were fed a diet free of vitamin C and low in the vitamin B complex. Gottlieb reported 4 cases of 'bachelor scurvy' associated with a high color-index anemia. In the 2 cases in which gastric analyses were done, hydrochloric acid was present in normal amounts. The patients were given a hospital diet plus ascorbic acid, the anemia responding satisfactorily. Jennings and Glazebrook reported 2 cases of adult scurvy with anemia. In the first case there was definite macrocytic anemia as well as achlorhydria. Constant reticulocytosis, polymorphism, anisocytosis, and leukopenia were also observed. The bone marrow was hyperplastic with megaloblasts present. The anemia failed to respond to either liver or iron and was cured with ascorbic acid. The second case was characterized by normochromic normocytic anemia and megaloblastic bone marrow. A complete response was obtained with ascorbic acid. Mettier, Minot, and Townsend found anemia in 8 of 9 cases of scurvy in adults. A normoblastic bone marrow was found in the 2 cases in which marrow examination was performed. The anemia responded to foods rich in vitamin C with prompt reticulocytosis and rapid regeneration of blood. Large doses of iron and liver had no effect. Others have reported that iron and liver preparations were useless and that the anemia responded to either pure ascorbic acid or to vitamin C-containing foods.

There are several reports which fail to demonstrate a relationship between ascorbic acid and erythropoiesis. The most convincing of these is the report of Crandon, Lund, and Dill. A normal active adult placed himself on a vitamin C-free diet supplemented with the other known vitamins for a period of 6 months. Although many manifestations of ascorbic acid deficiency appeared, anemia did not develop in spite of a blood loss from venesection of over 6 liters. Lozner made observations in 5 patients with "presumptive vitamin C deficiency and anemia." In 4 of these, regeneration of hemoglobin took place spontaneously or in response to iron therapy alone. He concluded that "hemoglobin regeneration may occur in the absence of reduced ascorbic acid from the blood by chemical test." Actually only plasma ascorbic acid determinations were made. It is now generally recognized that a low level of vitamin C in the plasma does not necessarily indicate scurvy. It should be noted that one of Lozner's cases was complicated by a bleeding peptic ulcer, another by alcoholic pellagra, and a third was a woman with achylia, a urinary tract infection, and a history of nine pregnancies. Croft and Snorf have been quoted as presenting evidence that synthetic vitamin
C is not the antianemic factor in scorbutic anemia. Actually the patients studied by them had none of the signs or symptoms of scurvy. The group consisted of 100 hospital patients taken indiscriminately who suffered from peptic ulcers, cirrhosis of the liver, infections, and a wide variety of other diseases. In all the ascorbic acid in the plasma was low. There is no reason to believe that the anemia which they treated unsuccessfully with ascorbic acid was due to scurvy. Other adequate and more likely causes for anemia were present.

Liu, Chu, Yu, Hsu, and Cheng selected 16 anemic children from an institution. One-half were treated with ascorbic acid and one-half with ferrous carbonate. Eleven of the 16 cases according to the figures presented had macrocytic anemia. The iron-treated group responded with a marked rise in the erythrocyte count and hemoglobin although no reticulocytosis was noted. The group treated with ascorbic acid failed to respond. They concluded that the anemia was not due to a lack of vitamin C but related in all probability to a concomitant iron deficiency. It is strange that of the 8 patients reported as responding to iron only 1 had a microcytic anemia and 6 had macrocytic anemias. The absence of a reticulocytosis is also unusual. Whether there was increased capillary fragility in these cases is not clearly stated. Ralli and Sherry maintained an anemic, scorbutic patient on a diet devoid of vitamin C but adequate in all other respects for 52 days. There was no further reduction in either the number of red cells or in the percentage of hemoglobin. Ascorbic acid therapy was discontinued on another patient after a response had occurred; after 45 days on a scorbutic diet no anemia appeared. Kenney and Rapoport observed 3 scorbutic infants in whom there was no response to ascorbic acid but a good response to iron. Several authors have observed reticulocytosis and hemoglobin regeneration on a vitamin C-free diet.

The literature dealing with the relationship of ascorbic acid to hemoglobin and red cell formation is difficult to evaluate. With the data available at the present time it would not be wise to draw dogmatic conclusions. Clinical scurvy is obviously complicated in many cases by multiple deficiencies, infection, and hemorrhage. It should not be expected that the blood picture would be uniform or that the therapeutic response to a certain substance would be constant in all cases. If several factors are deficient, the administration of only one of these should have little effect on red blood cell regeneration.

G. Other Vitamins.——1. Pantothenic acid.——The presence of a moderate normocytic anemia in dogs maintained on a diet deficient in filtrate factor II (pantothenic acid) was noted by Fouts, Helmer, and Lepkovsky. Wintrobe et al. noted a moderate normocytic, normochromic anemia in 13 of 18 pigs raised on a highly purified diet deficient in pantothenic acid. In 7 of the 13 animals the volume of packed red cells ranged between 35 and 40 cc. per 100 cc. of blood (normal 41 to 51). In the remaining 6 animals the volume of packed cells ranged between 24 and 33 cc. In the 2 animals receiving treatment with calcium pantothenate the anemia became less pronounced, the volume of packed red cells rising from 25 to 38 cc. in 1 animal and from 33 to 39 cc. per 100 cc. in the other.

It has been reported that a deficiency of pantothenic acid in rats may result in
anemia, granulocytopenia, and bone marrow hypoplasia. These changes, however, were not produced in all of the animals. Although the inclusion of pantothenic acid in the diet almost completely prevented the appearance of anemia, it did not do so completely and therapy with this vitamin was even less successful once the deficiency was established. Some of the rats responded to \textit{L. casei} therapy. It seems that a deficiency of pantothenic acid predisposed the animals to \textit{L. casei} factor deficiency and that the prophylactic administration of pantothenic acid prevented the development of this complication. The therapeutic administration of pantothenic acid at times seemed to arrest the deficiency of \textit{L. casei} factor. There was also evidence that instead of, or in addition to, \textit{L. casei} factor deficiency there developed a deficiency of an unidentified vitamin.

2. \textit{Choline}.—A marked decrease in hemoglobin and volume of packed red cells in dogs with a severe choline deficiency has been reported by McKibbon, Thayer, and Stare.\textsuperscript{236} Treatment with choline failed in most cases to restore the blood entirely to normal but this may have been due to marked and irreversible damage to the liver.

3. \textit{Biotin}.—Data have been presented by Ruegamer, Michaud, Elvehjem, and Hart\textsuperscript{231} which indicate that biotin is necessary for the production of hemoglobin values greater than 14 grams per cent in dogs maintained on a highly purified alcohol extracted casein ration. Without biotin in the diet it was found that there was a plateau in hemoglobin values at 11 to 14 grams per cent and severe achromotrichia developed. No evidence that biotin deficiency in the rat has a significant effect in red blood cell or hemoglobin regeneration has been found.\textsuperscript{1}

4. \textit{Monkey anemia factor}.—Monkeys maintained on a purified diet supplemented with a folic acid concentrate and all of the known vitamins except riboflavin develop a hypochromic anemia and a leukopenia, as mentioned previously.\textsuperscript{6} Upon the administration of riboflavin a definite increase in hemoglobin, red cell and white cell count in the blood has been observed but a plateau below normal is soon reached. Iron, “pseudo-pyridoxine,” 1:20 liver powder, extracted liver residue, and increasing the casein level to 24 per cent have proved ineffective in restoring the blood picture to the normal level. A factor (or factors) found in whole liver substance was necessary for optimal blood regeneration. Thus, it would seem that monkeys require an additional factor for erythropoiesis. This factor (or factors) is evidently labile since commercial extraction procedures used to prepare 1:20 liver extract and extracted liver residue from whole liver destroyed nearly all activity. Fresh liver was found to be a more potent source than whole liver powder. Lyophilized liver retained all of the active principle of fresh liver.\textsuperscript{232}

5. \textit{Pigeon anemia factor}.—The presence of an anemia was noted in early experiments in which beriberi was produced by maintaining the birds on a diet of polished rice. Recent experiments using a purified diet have demonstrated that the anemia does not respond to thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenic acid, choline, para-aminobenzoic acid, and inositol.\textsuperscript{233–236} The anemia does respond to yeast or crude liver extract. The possibility that the anemia is due to a deficiency of folic acid has not been ruled out although folic acid is adsorbable on fuller's
earth at pH 1, whereas the pigeon factor is very poorly adsorbed on fuller's earth at pH 4.0. Whether the dissimilarity in adsorption may be due to the difference in pH is not known.

6. Guinea pig anemia factor.—Severe anemia and leukopenia have been reported in guinea pigs fed succinylsulfathiazole and maintained on purified rations supplemented with the known vitamins, linseed oil meal, and solubilized liver. Thus there is convincing evidence that guinea pigs require an additional factor (or factors) for the maintenance of normal leukocyte and hemoglobin levels. This factor (or factors) is supplied in part by alfalfa, grass juice powder, crude liver extracts, and to a lesser extent by yeast. Folic acid does not appear to be the active substance since 4 per cent solubilized liver was included in the basal ration.

III. AMINO ACIDS

It is understandable when one considers the relative size, complexity, and amino acid content of the globin fraction of the hemoglobin molecule that a dietary deficiency of protein or of a specific amino acid might result in anemia. Because of the difficulty in preparing a diet deficient in a single amino acid only a few experiments of such a nature have been conducted. These include deficiencies of tryptophan, lysine, and phenylalanine. Most of the work hitherto reported has been concerned with the rate of hemoglobin regeneration in animals made anemic by phlebotomy. Unfortunately, by this method it is not possible to study the characteristics of the anemia.

A. General Protein Deficiency.—Rats fed a diet abnormally low in protein but adequate in all other respects develop anemia which responds to protein therapy. The anemia is characterized by a distinctly subnormal hemoglobin content of the blood, a normal erythrocyte count, and a mild reticulocytosis. Metcoff, Favour, and Stare have found that in acute protein deficiency in the rat there is an associated hemoconcentration and a significant decrease in the total circulating hemoglobin as the result of a reduction in the total blood volume. In chronic protein deficiency they found that although the hemoglobin concentration was unaltered the total circulating hemoglobin was significantly decreased. They point out that only if the total circulating hemoglobin is determined and adjusted to a unit of surface can the true severity of the anemia be appreciated. It has also been shown that dogs maintained on a diet deficient in protein show a progressive fall in the total red cell volume as well as in the red cell count and hemoglobin concentration. The rate of blood regeneration in rats and in dogs made anemic by repeated hemorrhage is accelerated by an adequate dietary intake of protein. Bethell has suggested that the anemia which occurs in pregnant women and which is characterized by a normal color index and by red cells of normal or increased volume is due to a protein deficiency. Orten and Keller have found that the excretion of porphyrin in the feces of rats is diminished in animals fed a diet low in protein.

B. Composition of Globin.—The minimal molecular weight of hemoglobin has been shown to be about 16,700 and studies of the osmotic pressure and of the sedimentation constant have shown that the true molecular weight of
anhydrous hemoglobin is close to 66.700, indicating that hemoglobin is composed of four globin units. According to Svedberg globin is a polydisperse protein and does not have a definite molecular weight. There is evidence in the literature suggesting both the existence and the absence of more than one hemoglobin in the same animal.

The amino acid composition of globin has been studied in several species and has been shown to contain all of the essential amino acids as well as many of the non-essential ones. It seems that the molecular ratios of tryptophan, tyrosine, arginine, histidine, and lysine are constant for all mammalian hemoglobins, being 2:3:8:9:9.

Table 4—The Approximate Amino Acid Composition of Various Hemoglobins, in Per Cent

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Horse</th>
<th>Man</th>
<th>Sheep</th>
<th>Bovine</th>
</tr>
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<tbody>
<tr>
<td>Leucine</td>
<td>15.12</td>
<td>17.28</td>
<td>16.4-17.7</td>
<td>14-16</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.66</td>
<td>0.81</td>
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<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>7.76</td>
<td>8.05</td>
<td>7.45</td>
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<td>4.2N</td>
<td>3.95</td>
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<td></td>
<td>7.75</td>
<td>8.05</td>
</tr>
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<td>Valine</td>
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<td>10.1N</td>
<td>7.7-8.1</td>
<td>7.5-10.3</td>
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<td>Tyrosine</td>
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<td>2.065</td>
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<td>Alanine</td>
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<td>9.96</td>
<td>8.7-9.2</td>
<td>8.5-8.9</td>
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<td>16.66</td>
<td>16.16</td>
<td>15.76</td>
</tr>
</tbody>
</table>

The superior numbers refer to the bibliographical source.

respectively, and that many of the differences in mammalian globins can be accounted for by the content of total sulfur, cystine, and methionine. The approximate percentage of amino acids in globin for several species is given in table 4.

C. Role of Amino Acids.—1. General.—The effects of the administration of various amino acids on the hemoglobin content of rats maintained on a low protein diet consisting of 3.5 per cent lactalbumin have been studied by Orten and Orten. No consistent, sustained increase in hemoglobin values occurred following supplementation with any of the ten essential amino acids or with glycine, cystine, glutamic acid, proline, or tyrosine. They interpreted these results as evidence that no single amino acid can be regarded as a "key" amino acid in hemoglobin synthesis. It is logical to assume that if more than one amino acid is...
lacking, then hemoglobin synthesis will not proceed until all of the missing amino acids are supplied. They conclude that "a combination of amino acids in as yet undetermined proportions is essential for the in vivo fabrication of the hemoglobin molecule."

Whipple and Robscheit-Robbins have shown that certain amino acids given to dogs made anemic by repeated hemorrhage cause an increase in new hemoglobin production over the basal level. Threonine, glycine, glutamic acid, aspartic acid, cystine, histidine, phenylalanine, and proline caused an increase in hemoglobin output of 23 to 24 grams above the control levels for a 2 week period. Leucine, methionine, lysine, and tryptophan caused an average increase of 20 grams and alanine, tyrosine, valine, isoleucine, arginine, and hydroxyproline increased hemoglobin output 10 to 17 grams over the control level for the 2 week period. There was no correlation between the quantity of an amino acid found in globin and its hemoglobin-regenerating activity.

In rats made anemic by the injection of phenylhydrazine Yashoda found that histidine accelerated the formation of erythrocytes and hemoglobin and that tyrosine was ineffective in this capacity. Brand and Stucky273 found that rats bred from mothers subsisting on a low protein (cystine-deficient) ration recovered from their "milk anemia" and showed good growth when glutamic acid was added to their iron supplement; whereas those from which glutamic acid was withheld but the iron added, died.

In dogs made anemic and hypoproteinemic Whipple and his group271-276 have shown that globin can be readily formed from pure amino acid mixtures, plasma, serum digests, casein, hemoglobin given intraperitoneally, and hemoglobin digests given by mouth. The body seems to give preference to hemoglobin production over serum protein production.

2. **Tryptophan.**—For some time it has been known that tryptophan accelerates recovery and causes a prompt reduction in the reticulocytosis seen in anemia caused by phenylhydrazine.280-283

By acid hydrolysis of casein it has been possible to prepare a protein which is almost totally deficient in tryptophan. This method has enabled workers to study the chemical and morphological characteristics of the anemia. Fontes and Thioville,284 Hamada,282 and Chin283 reported that rats maintained on a diet in which the protein was supplied in the form of an acid hydrolysate of casein became anemic and that this anemia was relieved by the administration of tryptophan. Alcock286 could not confirm these findings but Albanese et al.286 have pointed out that Alcock's experiments were not of sufficiently long duration and that the diet may not have been entirely free of tryptophan. Albanese et al.286 maintained rats on a diet in which the protein consisted entirely of acid hydrolyzed casein and reported that a mild normocytic, hypochromic anemia developed in 10 of 16 animals. The anemia in all cases responded to tryptophan therapy.

The anemia occurring in swine maintained on acid hydrolyzed casein as the only source of protein has been studied by Cartwright et al. The anemia was normocytic, or slightly microcytic, and normochromic. It developed early and was slowly progressive. There was no evidence of increased hemolysis as determined
by icterus index and qualitative urobilinogen determinations. The serum iron levels remained within normal limits even at the height of the anemia and there was no reticulocytosis. Terminally, that is a week or so prior to death, leukopenia appeared and persisted in all 3 of the animals studied. Differential leukocyte counts revealed no consistent or significantly greater reduction in the number of cells of one series of leukocytes as compared with another. Blood platelets were not reduced in number. A hypoproteinemia developed simultaneously with the anemia. When the hypoproteinemia became severe peripheral edema appeared. The bone marrow in 2 of the animals was normoplastic and in the third hypoplastic. Hemosiderosis of the tissues was not present. The effect of adequate doses of tryptophan in restoring the animals to normal was not determined. Until sufficient tryptophan is available for this to be done it cannot be conclusively stated that the changes described were due to a deficiency of this amino acid.

As discussed earlier in this review pyridoxine-deficient rats, dogs, and swine excrete certain metabolites of tryptophan in the urine. In order to ascertain the effects of a low tryptophan intake on the course of pyridoxine deficiency Cartwright et al. placed swine on a diet unsupplemented by pyridoxine and in which the protein was supplied in the form of acid hydrolyzed casein. It was concluded that a low intake of tryptophan retards the course and diminishes the severity of the nutritional disorder due to pyridoxine deficiency. This is in accord with the work of others in rats, mice, and dogs.

Since a lack of either tryptophan or of pyridoxine leads to the development of anemia and since there is a disturbance of tryptophan metabolism in pyridoxine deficiency the question arises whether pyridoxine anemia may be due to a lack of properly metabolized tryptophan. If such were true the two anemias should be similar. They are, however, quite different. The anemia of pyridoxine deficiency is microcytic and slightly hypochromic and is accompanied by an elevated serum iron, hyperplastic bone marrow, and hemosiderosis of the liver, spleen, and bone marrow. The anemia associated with the feeding of acid hydrolyzed casein is essentially normocytic and normochromic, the bone marrow appears to be hypoplastic, the serum iron level is normal and there is no hemosiderosis of the tissues. The two deficiencies are markedly different. Tryptophan deficiency causes cessation of growth and is accompanied by marked hypoproteinemia and edema. Pyridoxine deficiency causes only limitation of growth and the quality and quantity of the serum proteins are unaffected. The two anemias are compared in table 5.

3. Lysine.—It is known that deaminization of casein destroys the entire content of lysine, about half of the histidine, and a portion of tyrosine. Hogan and Ritchie reported that rats maintained on deaminized casein develop anemia and splenomegaly. When casein was added to the deaminized casein ration the anemia failed to develop. Smith and Stohlman studied the anemia and reported that after 10 to 15 days on the diet examination of the blood revealed anisocytosis, a moderate degree of reticulocytosis, polychromatric macrocytes frequently containing one or more Howell-Jolly bodies, and a substantial degree of anemia. As the disease progressed the anemia became more pronounced, the red cells falling as low as 2 million and the hemoglobin to 20 per cent. The blood smears in the
advanced stage showed many poikilocytes, microcytes, macrocytes, polychromatic cells, "megaloblasts," and numerous Howell-Jolly bodies. The reticulocyte count ranged from 5 to 25 per cent. Immature white cells such as myelocytes and myeloblasts appeared in various numbers. The anemia was usually macrocytic and slightly "hyperchromic." By boiling deaminized casein with alcoholic sodium hydroxide, or by reprecipitating the deaminized casein from aqueous alkaline solution, the anemia-producing factor could be destroyed to a considerable extent. The intraperitoneal injection of the alcohol-soluble fraction of a hydrochloric acid hydrolysate of deaminized casein reproduced the anemia and the authors concluded that the anemia was not a deficiency disease but was due to the presence of a toxic substance in the deaminized casein. Hogan and co-workers extended their experiments and found that lysine was the antianemic agent in the deaminized casein-anemia syndrome. However, it was necessary to administer 2 to 4 times the normal requirement of lysine to prevent the anemia. These authors speculated that lysine was required to detoxify the deaminized casein. Attempts to isolate lysine from the urine of rats supplied with this amino acid failed.

Gillespie, Neuberger, and Webster have presented evidence that lysine deficiency in rats results in an anemia. They maintained rats on a diet in which the protein was supplied in the form of zein, a protein deficient in both lysine and tryptophan. One group was given a daily supplement of tryptophan and lysine. A second group received only tryptophan. In the group receiving lysine the average red cell count and hemoglobin were 8.76 million and 14.9 Gm. per cent, respectively. In the group receiving no lysine the average red cell count was 7.10 million and the average hemoglobin 11.1 Gm. per cent. Thus there was a mild anemia with a slightly greater average reduction in hemoglobin than red cells. In a third group of animals it was demonstrated that the anemia was not due to inanition.

Muller has reported that when lysine is injected intravenously into pigeons a

<table>
<thead>
<tr>
<th>Determination</th>
<th>Casein Hydrolysate</th>
<th>Pyridoxine Deficient</th>
<th>Control</th>
</tr>
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<tr>
<td>R.B.C., millions cu. mm.</td>
<td>4.58</td>
<td>6.50</td>
<td>7.93</td>
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<td>HBG. Gm.%</td>
<td>8.2</td>
<td>8.9</td>
<td>14.0</td>
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<td>Hematocrit, cc.</td>
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<td>29.3</td>
<td>46</td>
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<td>MCV, cu.</td>
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<td>41</td>
<td>58</td>
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<tr>
<td>MCH, (\mu g)</td>
<td>18</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>MCHC, (\mu g/\text{L})</td>
<td>34</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>W.B.C., cu. mm.</td>
<td>5500</td>
<td>17,500</td>
<td>18,000</td>
</tr>
<tr>
<td>Serum Iron, (\mu g/\text{L})</td>
<td>131</td>
<td>374</td>
<td>142</td>
</tr>
<tr>
<td>Total Protein, Gm. %</td>
<td>3.88</td>
<td>6.57</td>
<td>6.33</td>
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<tr>
<td>Bone Marrow</td>
<td>N</td>
<td>H</td>
<td>N</td>
</tr>
<tr>
<td>Hemosiderosis</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

N = Normoplastic.
H = Hyperplastic.
reticulocytosis occurs, this reticulocytosis being caused by a stimulation and proliferation of the red blood cells in the bone marrow with an extension of the blood-forming marrow tissue.

4. Phenylalanine.—Maun, Cahill, and Davis\textsuperscript{254} fed synthetic diets of crystalline amino acids, crystalline vitamins, fats, dextrin, and salts to young rats. When phenylalanine was omitted from the amino acid mixture for 18 days the hemoglobin ranged from 7.4 to 14.0 Gm. per cent, with an average of 9.9 Gm.; animals on the complete diet had hemoglobin levels ranging from 13.7 to 15.8 Gm., with an average of 14.7 Gm. Hemosiderosis of the tissues was not noted.

5. Glycine.—Evidence for the direct utilization of glycine for the formation of the pyrrole rings of protoporphyrin in humans has been presented by Shemin and Rittenberg.\textsuperscript{297} Glycine containing N\textsuperscript{15} was consumed by a human subject. Hemin was then isolated at various intervals and found to contain significant quantities of N\textsuperscript{15}. Previous experiments in which leucine\textsuperscript{298} and ammonia\textsuperscript{299} labeled by N\textsuperscript{15} were fed to rats gave no indication that these substances were directly concerned with porphyrin synthesis. It has been suggested that tryptophan, proline, and hydroxyproline are precursors of protoporphyrin. There is no direct evidence to confirm this.

6. Isoleucine.—Orten, Bourque, and Orten\textsuperscript{300} have presented evidence that isoleucine deficiency in the rat results in a mild to severe anemia. The animals were fed a synthetic diet containing purified human or beef globin as the protein. Both of these proteins have been shown to be deficient in isoleucine. Supplementation of either type of globin with isoleucine resulted in the maintenance of a normal concentration of hemoglobin in the blood. The subsequent removal of isoleucine was followed by the development of anemia and death of the animals. The finding that isoleucine is needed for hemoglobin formation in the rat raises an interesting question. Either the globin of rat hemoglobin, unlike that of human or beef hemoglobin, contains isoleucine or isoleucine must be concerned in the formation of some intermediate compound.

IV. MINERALS

A. Iron.—That iron is a constituent of the hemoglobin molecule\textsuperscript{301, 302, 304} and that a deficiency of iron gives rise to an anemia are both well established. The magnitude of the literature on various aspects of iron metabolism is great, yet there are large gaps in our knowledge of certain phases. In recent years there have been several reviews on the subject.\textsuperscript{306–311} However, since these reviews were published the fundamental concepts of iron metabolism have changed greatly.

1. Availability.—Dietary iron is of two types, organic and inorganic.\textsuperscript{315} The organic iron is present principally in the form of iron porphyrin compounds. The ability of inorganic iron salts to cure iron deficiency anemia has been well demonstrated in many different species. On the other hand, it has been shown that organically bound iron is very poorly utilized when given orally. Bunge’s theory\textsuperscript{313} that iron occurs in food as complicated organic compounds which are absorbed and assimilated as such and are built directly into hemoglobin has not been confirmed by modern studies. Organic iron, if it is to become available, must be
converted to an ionizable form. In the case of hematin it has been shown that it is less than 25 per cent utilized and this utilization appears to be dependent upon the amount of decomposition by intestinal bacteria.\textsuperscript{315, 316} It has not been demonstrated that a preformed iron compound is absorbed and used as such in building the hemoglobin molecule. On the available information it would seem that the body's need for iron can be entirely satisfied by inorganic iron compounds.

Hill\textsuperscript{314} has shown that \(\alpha\)-dipyridyl reacts with ionizable ferrous iron to form an intense red compound. The reagent does not react with hematin or other organically bound iron compounds. Elvehjem and co-workers\textsuperscript{317, 318} have shown that there is a moderately good correlation between the biologically active iron in foodstuffs and the iron determined with \(\alpha\)-dipyridyl following reduction of the ferric iron. Tables of the "available" iron content of foodstuffs have been made using this method. Their value is somewhat limited for as Hahn and Whipple\textsuperscript{319} pointed out, the term "available iron" as determined by the dipyridyl test has little physiological significance. An iron salt which is rated as 100 per cent available by the dipyridyl test may be only 40 per cent physiologically active.

2. Absorption.—Iron is absorbed chiefly in the duodenum, but the stomach and the whole of the small intestine may take part under certain circumstances.\textsuperscript{320–323} It is doubtful that any absorption takes place from the colon.\textsuperscript{325} It is absorbed by the tips of the villi of parts of the duodenum and from there is passed into the portal circulation and is carried to the liver. Moore et al.\textsuperscript{324} have shown that iron is not transported by the thoracic duct lymph.

There are many factors affecting the absorption of iron. By measuring the serum iron increase following the oral administration of iron in various forms and under various conditions Moore and co-workers\textsuperscript{322, 324} could define many of these factors. Whipple and his group, working with an entirely different method, have been able to define others. The present knowledge concerning the absorption of iron may be summarized as follows:

a. Type of iron.—It is generally believed that iron is absorbed in the soluble, ionizable, ultrafiltrable, ferrous form. The absorption of ferric compounds is generally less than that of ferrous compounds and is dependent on the capacity of the intestinal contents to reduce them. The nature of the anion, except as it influences the ease of ionization, is relatively unimportant. Organically bound iron as stated above is very poorly absorbed.

In the past there has been disagreement between the clinical and animal investigators as to whether the ferrous form is more readily absorbed than ferric iron. Using the radioactive iron technic it has been demonstrated in two different laboratories that human subjects absorb ferrous iron more efficiently than ferric iron.\textsuperscript{325, 326} Dogs absorb both valence forms well although in some instances there is a greater uptake of ferrous than ferric iron. Rats absorb radioactive ferrous and ferric iron equally well.\textsuperscript{327} Thus there is a species difference and this probably accounts for the disagreement between clinical and animal investigations. Moore et al.\textsuperscript{325} offer three possible explanations for the greater absorption of bivalent iron for the human being: (1) only ferrous iron may be absorbed and all trivalent iron may have to be reduced before it can be taken up by the body; (2) both forms
may be absorbed but to an unequal degree; and (3) ferric iron may be made less available for absorption because it more readily forms complex insoluble compounds within the intestinal tract.

b. Amount of iron.—The height of the serum iron increase is directly proportional to the amount given up to that point at which intestinal irritation is great enough to interfere materially with intestinal motility.

c. Gastric acidity.—The free gastric hydrochloric acid performs two functions: (1) reducing, ionizing, and dissolving the iron, and (2) delaying the formation of insoluble and undissociated iron compounds which may occur above pH 5. For these reasons any factors which cause increased alkalinity diminish iron absorption.

d. Conditions in the duodenum.—The pH of the duodenal contents likewise affects the ionization and solubility of the iron compounds. The presence of certain reducing substances in the diet aids the absorption of iron. Fisher and Peabody\(^328\) have shown that liver extract has marked reducing properties. Oxidation of ferrous iron is effectively prevented by liver extract and 50 to 95 per cent of ferric iron added to it is reduced. The reducing properties of ascorbic acid are well known.\(^324\) Freeman and Ivy\(^329\) have shown that calcium carbonate, aluminum hydroxide, and to a lesser extent magnesium trisilicate reduce iron retention in anemic rats. The absorption of iron from the intestinal tract is inhibited when iron is administered with relatively large doses of mucin in cases of chronic hypochromic anemia.\(^33\)

e. Ca:P ratio and vitamin D.—There is ample evidence that excess calcium in the diet inhibits iron assimilation and causes a mild anemia.\(^331\) Furthermore, all investigators are agreed that iron utilization is affected by the Ca:P ratio in the diet but there is disagreement as to whether a high or low ratio is more favorable. Anderson, McDonough, and Elvehjem\(^334\) have shown that hemoglobin regeneration and iron storage in the liver were the greatest on the lowest Ca:P ratio and that, as the ratio was increased, there was a corresponding decrease in both hemoglobin regeneration and iron storage. These results have been confirmed by Fuhr and Steenbock\(^335\), \(^336\) but are contradictory to those of Day and Stein.\(^337\) The latter workers found that rats on a low mineral ration containing a relative excess of phosphorus develop a mild numerical increase in red cells together with a reduction of hemoglobin and that this is prevented by increasing the calcium in the diet. The action of calcium is explained by its ability to bind phosphorus, "thus permitting the dietary iron to be used for hematopoiesis instead of being excreted, presumably as a phosphate."

Where the discrepancies lie between these two experiments is not clear but the important fact is that neither a high nor a low Ca:P ratio is incompatible with normal hematopoiesis if the diet contains sufficient iron, for the action of these minerals is not directly on hemoglobin formation but rather on iron absorption. When this is appreciated the anemia accompanying rickets is better understood.

Fuhr and Steenbock\(^338\) have demonstrated in rats that vitamin D improves the storage of iron and the formation of hemoglobin when the diet contains optimal amounts of calcium and phosphorus. A similar stimulus to hemoglobin formation was noted by Day and Stein.\(^337\)
Selective absorption.—McCance and Widdowson introduced the idea that the intestine controls the amount of iron absorbed. Rohscheit-Robbins and Whipple have shown that the efficacy of iron feeding depends upon the actual need of the body for the element. Recent studies by Whipple and co-workers using a radioactive isotope of iron have been enlightening. They showed that dogs made anemic by frequent bleeding while on a low iron diet had 4.1 to 12.7 per cent of the ingested radioactive iron in the tissues and blood while normal nonanemic dogs had only 0.08 to 0.24 per cent accountable. These experiments were extended and it was concluded that the mucosa of the gastrointestinal tract has the power to reject or to accept iron. These studies were then carried to human beings and it was found that when there is need of the body for iron as in pregnancy and iron deficiency states, the iron intake is increased above normal, whereas in disease states in which the iron stores are known to be very abundant as in pernicious anemia, hemochromatosis, familial hemolytic icterus, and Mediterranean anemia, there is very little absorption. They concluded that "reserve stores of iron in the body, rather than anemia, control iron absorption. This control is exerted on the gastrointestinal mucosa which can refuse or accept iron under various conditions."

A possible fallacy in the experiments of Hahn and Whipple on the absorption of iron in various anemic states in man must be pointed out. The amount of radioactive iron appearing in the red cells was used as an indication of the amount absorbed. In anemic states other than iron deficiency it is possible and even likely that owing to a diminished rate of hemoglobin formation much of the absorbed iron does not appear in the hemoglobin. The amount of iron appearing in the hemoglobin is not necessarily an indication of the amount absorbed but rather of the amount utilized. If hemoglobin cannot be formed in cases in which iron is not the limiting factor then iron, even though absorbed, cannot be utilized. In such cases the amount of iron appearing in the red cells would give a false impression of the amount absorbed.

Subsequent studies by Hahn and co-workers as to the precise mechanism by which the gastrointestinal mucosa accepts and refuses iron have revealed that an acute severe anemia or anoxia of 24 hours' duration does not significantly increase iron absorption. After approximately 7 days, at which time the iron stores are depleted, absorption becomes active. These workers speculate that there is an "acceptor compound" in the mucosa which is capable of taking up iron and passing it on to the plasma, and that the plasma iron concentration may regulate the degree of iron saturation of this acceptor and consequently its ability to pass iron from the intestinal lumen to the plasma. They further speculate that once iron reaches the plasma it is combined with protein in a combination which is not sufficiently loose to allow transfer of the iron in the reverse direction through the acceptor. "Thus this acceptor together with the action of the plasma might act as a valve mechanism allowing the body to obtain iron when needed and conserve what it has for future needs."

Granick has discovered that the content of ferritin in the gastrointestinal mucosa is relatively high and that it increases in response to iron feeding.
On this basis he has suggested the following modification of the hypothesis of Hahn et al.: Iron is absorbed into the mucosal cells in the ferrous form where it is stored as ferritin (a combination of the protein apoferritin with ferric hydroxide). There is an equilibrium in the cell between ferrous iron and the ferric iron stored in ferritin, the cell being in a state of "physiological saturation" with respect to ferrous iron. As the concentration of the plasma iron falls, ferrous iron is removed from the mucosal cell, resulting in a diminution of ferritin iron in the mucosa. When the ferritin iron has diminished to a point where the cell is no longer saturated with respect to ferrous iron, more iron is absorbed by the cell.

Moore and his group\textsuperscript{322} have questioned the selective absorption theory and cite as evidence against this theory the fact that some patients with hypochromic anemia before therapy do not have greater iron absorption curves than after therapy. They believe that a more likely explanation would be that iron is absorbed by a simple process of diffusion through the interepithelial spaces into the bloodstream and that the magnitude of the concentration of the gradient of iron between the intestinal lumen and blood plasma determines the amount of iron absorbed. As pointed out by Hahn et al., this theory does not explain the fact that smaller doses of iron are more efficiently absorbed than larger ones. And as noted by Moore et al., it is in contrast to the histological experiments of Höber\textsuperscript{316} which tend to show that iron is absorbed intra-epithelially.

Since Moore et al. admit that iron-deficient animals absorb iron more readily than those with adequate stores and that there is no histological evidence for simple diffusion through the interepithelial spaces, and since Hahn et al. now believe that the serum iron level may play a role, it would seem that the divergence between these two views is not great.

8. Other factors.—There is suggestive evidence that pyridoxine may regulate iron absorption. This has been discussed under pyridoxine. It has been shown that the average retention of iron in children is slightly lower during periods of high thiamine intake than during control periods.\textsuperscript{417} These results, however, could not be confirmed in rats on a well controlled experiment.\textsuperscript{515} The amount of food consumed affects iron absorption. With a constant intake of iron, an increase in consumption of food results in a lower retention of iron, and a decrease results in a higher retention.\textsuperscript{417}

3. Transportation.—There is now ample experimental evidence that there is a fraction of iron in the plasma which is in a nonhemoglobin form.\textsuperscript{325,349–351} Using radioactive iron Yoshikawa, Hahn, and Bale\textsuperscript{355} have shown that about 95 per cent of the plasma iron fraction is bound to protein and of this only 15 per cent is bound to the globulin fraction. However, 90 per cent of the plasma iron is not precipitated by trichloroacetic acid and therefore must be very weakly bound. Whether there is true salt formation or whether the protein tie-up is one of adsorption has not been determined. \textit{In vitro} studies have shown the serum iron is in the ferric state and as such is non ultrafiltrable but upon reduction to the ferrous state becomes ultrafiltrable.\textsuperscript{358} This is known as the "Barkan phenomenon." The true state of plasma iron \textit{in vivo} is unknown.

The significant fact concerning plasma iron is that it has been demonstrated
to function as transport iron. Following a large dose of iron salts there is a prompt rise in the plasma iron fraction. The increase is apparent within the first half hour, reaches its maximum in 2.5 to 3 hours, and falls to the basal level in 12 to 18 hours. It is interesting that transportation and utilization are very rapid processes. Radioactive iron has been shown to find its way into red cells in as little time as several hours. No direct interchange between the plasma iron and the red cell has been demonstrated.

The normal range for plasma iron in human beings has been shown to be about 0.050 mg. to 0.180 mg. per cent. The values obtained by various workers are given in table 6. The average value for men is slightly higher than for women. There is essentially no difference between the amount contained in plasma as compared with serum. Moore, Minnick, and Welch found that the difference between minimum and maximum serum iron values in a given normal subject ranged within the daily cycle and during a 6 month period from 0.01 to 0.065 mg. per cent. Heilmeyer and Plötner have found variations of −10 to +19 micrograms per cent in 6 normal subjects during a period of 1 to 9 days. Similar observations have been made by others. Hemmeler has presented evidence that the serum iron is highest early in the morning, decreases gradually during the day, and rises during the night. Vahlquist found higher values at 6 P.M. than at 8 A.M. Evidence has been obtained for a menstrual periodicity in the serum iron in normal women. The serum iron has been found to be lower in old people than in young. There is evidence that physical exercise decreases the serum iron. Regnster induced fever in 3 subjects and noted a lowering of the plasma iron level.

The plasma iron level is affected by the rate of absorption of iron, the balance between that going to and from the tissues, and the equilibrium between the amount used for hemoglobin formation and that coming from hemoglobin catabolism. The values found in various pathological states are given in table 7. In general, in iron deficiency states it is uniformly low. Under conditions of decreased red cell formation (aplastic anemia, Mediterranean anemia, myelophthisic anemia,
and pyridoxine deficiency anemia in animals) it tends to be high. When the bone marrow is unusually active (acute hemorrhage and liver-induced remission in pernicious anemia) the plasma iron is low. In hemolytic states the plasma iron level is dependent upon the equilibrium between the iron released by hemolysis of red cells and the rate of uptake by the bone marrow.

Barkan has described a third form of blood iron, constituting 5 to 10 per cent of the total blood iron, which he termed the "leicht abspaltbare Bluteisen" fraction. This was so named because it was easily freed from its lightly bound state in the erythrocytes and plasma by acidification with weak acids. This finding has since been many times confirmed. When whole blood is incubated with 0.1 N hydrochloric acid 2.3 to 2.5 mg. per cent of iron is "easily split-off." Sulfuric and nitric acids split off more iron than does hydrochloric acid. The concentration of the acid also affects the amount of "easily split-off" iron obtained.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasma Iron</th>
<th>Reference</th>
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<tr>
<td>Iron Deficiency</td>
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</tr>
<tr>
<td>Mediterranean Anemia</td>
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<td>367</td>
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<td>Familial Elliptocytosis</td>
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<td>Pernicious Anemia</td>
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<tr>
<td>Nutritional Macrocytic Anemia</td>
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<tr>
<td>Sprue</td>
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<tr>
<td>Aplastic Anemia</td>
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<td>72, 350, 360</td>
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</table>
split-off" fraction constituted an organic, nonhemoglobinous form of iron and finally succeeded in establishing that the iron of iron-containing bile pigment compounds (pseudohemoglobins, verdohemochromogens), that is, open ring derivatives of hemoglobin, was "easily split off" by the action of dilute acids. He concluded from this that the "easily split-off" iron was due to the presence in the erythrocytes of verdohemochromogens. It has since been shown by Legge and Lemberg, and confirmed by Barkan and Schales and others, that the E fraction is an artefact arising from oxidation of the prosthetic group of the hemoglobin molecule by the O₂ evolved from oxyhemoglobin by acids. The E' fraction is partially attributable to a bile pigment-hemoglobin, a small part arises from blood catalase, and the remainder is due to the iron in the plasma.

<table>
<thead>
<tr>
<th>Table 8.—The Distribution of Iron in the Body as Determined in a Dog Weighing 20 Kilograms (Modified after Hahn306)</th>
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</thead>
<tbody>
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<tr>
<td>Hemoglobin iron</td>
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<tr>
<td>Myoglobin iron</td>
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<td>Parenchyma iron</td>
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<tr>
<td>Storage iron</td>
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<tr>
<td>ferritin</td>
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<tr>
<td>noncrystallizable ferritins</td>
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<tr>
<td>feritin</td>
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<tr>
<td>hemosiderin</td>
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<tr>
<td>Inorganic ferrous iron</td>
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</tbody>
</table>

4. Storage of iron.—Although the literature on this phase of iron metabolism is very extensive the storage of iron is poorly understood. There are numerous studies of the iron content of tissues as found under various conditions but little information is available concerning the chemical nature or physiological function of the various fractions. The exact total iron content of a specific tissue is dependent on many factors and is relatively unimportant. Many of the studies reported in the literature were done on tissues which were not blood free and with methods which have since been shown to be untrustworthy. The work of Whipple and his co-workers on the total iron content of tissues under various circumstances has been carefully done on blood-free tissues and with reliable methods and only this work will be summarized here. Subsequent papers confirm these findings but add little further knowledge on this phase of iron metabolism.

Present knowledge concerning the distribution of iron in the body of a dog of 20 Kg. weight (modified from Hahn306) is summarized in table 8.

The immobile fraction of body iron is made up of parenchyma iron and the iron
in myoglobin. This constitutes about 2.3 per cent of the total body iron. The Rochester group have shown that in the face of long-continued severe anemia and urgent need for iron from any available source, the dog does not draw on either the parenchyma iron of the tissues or on the muscle hemoglobin iron. Parenchyma iron is probably held tightly by the cells in such forms as cytochrome, catalase, peroxidase, and other cellular enzymes and is essential to the life of these cells. Myoglobin has been shown to be very similar to hemoglobin in respect to absorption, iron content, and elimination by way of bile pigments but may be differentiated by means of a specific precipitin reaction.

Whipple and his co-workers established the location of stored iron by injecting iron in dogs which had been made iron deficient for 2 to 3 months through repeated phlebotomy and a low iron diet. From these studies they concluded that the liver, spleen, and bone marrow are the principal sites of storage iron. Fifty to 70 per cent of iron injected intravenously can be shown to be stored in the liver and spleen. The liver contains the main bulk of the iron stored and is considered the most important organ in the conservation and utilization of iron in the body. The turnover in the liver is exceedingly rapid if there is a demand for the element for blood formation. However, the spleen contains the highest reserve stores of iron per 100 Gm. of fresh tissue. The iron content of the rib bone marrow runs parallel with that of the spleen as judged by iron storage following hemoglobin injections and depletions. There is some evidence that the manner in which iron arrives at the tissues affects its distribution. When radioactive ferric ammonium citrate was given intravenously most of it appeared in the liver. Iron liberated from hemoglobin by destruction of the red cells with phenylhydrazine was taken up by the spleen as well as by the liver. It has been shown that the kidney forms an important function in the conservation of iron. The glomeruli establish the minimal renal threshold for hemoglobin and prevent its escape. When this threshold is exceeded the tubular epithelium picks up the hemoglobin and saves it for future use. Finally when the tubular threshold is exceeded hemoglobin appears in the urine.

By use of the radioactive isotope it has been demonstrated that the normal stores of iron are not the first line of supply for blood formation when massive hemoglobin destruction has occurred. Rather the hemoglobin iron of the new cells is first derived from the cells broken down. The stores are drawn upon only if additional iron is necessary as a result of abnormal loss of the element from the body.

(To be continued in the next issue)