THE PRESENT STATUS OF FOLIC ACID

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WITH the recent demonstrations1-3 of the effectiveness of "folic acid" (synthetic L. casei factor) in the treatment of pernicious anemia, nutritional macrocytic anemia, and of sprue, it was considered sufficiently important to review the literature dealing with the various aspects of the work with this promising material. A review of this subject is timely not because the complete story of its discovery and identity can be told but rather because its clinical significance seems to urge the telling of its development as far as our present knowledge permits.

"Folic acid" is one of the newest members of the vitamin B complex. Like other better known members of this group, its discovery came from various laboratories with widely divergent approaches. In fact it is impossible to state with assurance that the divergent beginnings have all converged on the same substance, but at least in part they appear to have done so at this time. Thus, for the sake of clarity, we shall attempt to develop in the following pages each apparently unrelated group of studies individually until they begin to overlap. We shall then attempt to show how these studies have become integrated. No special effort to maintain proper chronology will be made.

1. GROWTH FACTORS FOR Lactobacillus casei AND Streptococcus lactis R

In 1940, Snell and Peterson4 published the tenth paper of a series entitled "Growth Factors for Bacteria" in which they revealed that certain lactic acid bacteria required extracts of plants or animals for growth. The basal medium was composed of purified hydrolyzed casein supplemented with riboflavin, pantothenic acid, niacin, pyridoxine, and tryptophan. Lactobacillus casei of Freudenreich was used as the test organism (Bergey classifies this bacterium as Lactobacillus helveticus). Yeast extract (Bacto) and solubilized liver fraction were both rich sources of the essential growth factor. It was found that a norite eluate factor was the most active fraction from these sources. Methods of concentrating the active principle were investigated and tests were made to determine some of its chemical properties. A compound of basic character was found which was labile to oxidation and was precipitated by many common basic precipitants. It showed some properties in common with naturally occurring purines. The following year Hutchings, Bohonos, and Peterson5 described a procedure used in further purifying the eluate factor from solubilized liver. Their material was not precipitated by well known basic precipitants so that the material was definitely less basic than that originally suggested. In fact studies with electrodialysis and esterification demonstrated...
clearly the acidic nature of the active principle. The destruction with nitrous acid and inactivation on acetylation or benzoylation suggested the presence of an amino group. The fact that the activity did not depend on the phosphorus content of the preparation led to the conclusion that a nucleotide was not an active part of the compound.

Earlier in the same year Stokstad had isolated a factor necessary for the growth of *L. casei* from solubilized liver by adsorption on norite and elution with 0.5 N ammonium hydroxide in 70 per cent methanol. Further purification consisted of fractional precipitation of the manganese salt of the factor with methanol. The final product had the properties of a nucleotide in that it contained nitrogen, phosphorus, and gave a positive Bial’s test for pentose. A negative Feulgen test proved it was not desoxyribose. On the basis of hydrolytic studies, Stokstad felt that the active principle contained a purine and a pyrimidine nucleotide. Guanine but not adenine was present. This was in line with observations reported by Snell and Mitchell in which both purine and pyrimidine bases when added to the culture medium were found to give better growth responses in certain lactic acid bacteria.

Also in 1941, Mitchell, Snell, and Williams obtained a highly concentrated growth factor for *Strept. lactis* R. from spinach. Because of the source of this material they suggested the name ‘folic acid’ and defined it as a growth factor for this particular organism. The growth of *L. casei*, however, was also stimulated by folic acid. No phosphorus was present in this factor and one-half maximal growth was obtained at a level of 0.00012 γ per ml., while Stokstad’s fraction required 0.014 γ per ml. Liver, yeast, and other substances were found to contain folic acid, and in preliminary studies on rats evidence suggesting intestinal synthesis was reported. Mitchell and Snell described a microbiological assay method for folic acid using *Strept. lactis* R. as the test organism. The amount of growth was measured by a thermoelectric turbidimeter or photoelectric colorimeter after 16 hours’ incubation. Standard curves were based on growth obtained between 20 and 200 micrograms of Wilson’s liver extract fraction ‘B’ (potency of one unit per microgram). The following year, 1942, Landy and Dickens described a microbiological assay method for ‘folic acid’ and other members of the vitamin B complex, using *L. casei* as the test organism. By the omission of certain single vitamins, no growth nor acid production was noted. The addition of increasing amounts of the missing vitamin up to a certain value gave proportionately increasing amounts of growth or acid production. The measure of growth could be made either by turbidimeter or by titration.

In 1943, Keresztesy, Rickes, and Stokes compared the amount of folic acid and norite eluate factor in various types of extracts and liver preparations and found that some of the materials were much more active for *Strept. lactis* R. than for *L. casei*. This observation was in contrast to that found for the extract of spinach, which had the same degree of activity for both organisms. A new substance was then obtained from an unstated source which effectively replaced folic acid in the growth of *S. lactis*, but was inactive for *L. casei*. One gamma of the new material had the same potency for *S. lactis* as 56 γ of folic acid, but 1 γ was less active for *L. casei* than 0.0004 γ of folic acid. At approximately the same time...
Stokstad isolated from liver and from yeast crystalline methyl esters which on hydrolysis yielded preparations of equal potency for \textit{L. casei}. The free acid of both fractions had the same absorption spectrum. When their microbiological potency was compared with the liver fraction B standard of Mitchell and Snell (see above), Stokstad's preparation from liver had a relative potency of 79,000 as determined with \textit{L. casei} and 78,000 with \textit{S. lactis R}. The yeast preparation had a potency of 75,000 with \textit{L. casei} and 38,000 with \textit{S. lactis R}. Thus the liver and yeast compounds differed from one another, and both seemed to be different from the growth factor isolated by Keresztesy et al. for \textit{S. lactis R}. Stokstad believed that the liver factor was identical with the compound obtained by Pfiffner et al. (see below).

A series of four papers appeared in 1944 describing the isolation and properties of folic acid obtained from spinach. Mitchell, Snell, and Williams concentrated a product which was 137,000 times more active for \textit{S. lactis R} than the standard liver product. The active principle was unstable to oxidation, reduction, acid, alkali, light, and heat and was highly reactive with many organic reagents. Attempts at crystallization were unsuccessful and all samples with potency exceeding 110,000 gave amorphous precipitates. In a special study of adsorption, Frieden, Mitchell, and Williams found that the elution of folic acid after adsorption on charcoal was much easier for crude preparations than for relatively pure solutions. This behavior was attributed to the presence of interfering substances which affected the manner of adsorption. In fact, adsorption isotherms indicated by a change in slope that the adsorption process was of a dual nature. This evidence, combined with certain chemical data, led Mitchell and Williams to the conclusion that most of the impurities present in their most concentrated preparations were of
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a similar nature to the active compound. Analyses suggested an empirical formula of C_{12}H_{12}O_{6}N_{4}. No sugar or polyhydroxy group was indicated, but the presence of a xanthopterin-like structural unit was suggested (fig. 1a). Diffusion coefficient measurements compared to those of compounds of known molecular weight gave a value for folic acid of 400 ± 0. In this paper the authors reported that a sample of folic acid with a potency of 75,000 had been tested by Norris for antianemic activity in trout. This test was suggested by an earlier report of Simmons and Norris\(^\text{18}\) that injections of 30–50 γ of xanthopterin from human urine brought about a cure of a nutritional anemia induced in Chinook salmon. Xanthopterin was just as effective in regenerating the blood as was liver extract. The folic acid, however, was only one-fifth as active as the xanthopterin since 10 γ per gram of fish was required for a good erythrocyte response. Mitchell\(^\text{19}\) obtained absorption spectra data to show that folic acid resembled xanthopterin. The light-absorbing structure was much more stable to light and acid treatment than the physiologically active compound. Difficulties in purification were thus attributed to inactive folic acid with physical properties only slightly changed.

Stokes, Keresztesy, and Foster\(^\text{20}\) found that the \textit{S. lactis} R. factor which they had recently isolated\(^\text{21}\) would not support the growth of \textit{L. casei} and other \textit{Lactobacilli}. If 1 ml. of the supernatant from a 24 hour broth culture of \textit{S. lactis} R. containing one hundred times the amount necessary for optimum growth (0.3 γ unit versus 0.003 γ units) was added to a culture of \textit{L. casei}, at least optimum growth was obtained. Thus SLR factor could be converted to "folic acid" by the streptococci and quickly enough to give identical growth curves when the two substances were compared. In fact these investigators found that all strains of bacteria capable of using the SLR factor could grow on "folic acid" to the same degree. This factor, however, unlike xanthopterin, did not give rise to "folic acid" when incubated with rat liver (see below). In their most recent paper Stokes and Larsen\(^\text{22}\) reported that resting cell suspensions of enterococci which required the SLR factor for growth, varied quantitatively in their ability to convert SLR factor to "folic acid." \textit{S. lactis} R. cells grown in a medium containing the SLR factor were more active than those grown with "folic acid" or thymine. Within the range of 0.01 γ to 2.0 γ per ml. of suspension, the concentration of SLR factor in the growth medium did not affect the conversion significantly. Cells from cultures nearing the logarithmic phase formed more "folic acid" than younger cells. The transformation was rapid since maximum folic acid was obtained within two hours and the amount was proportional to both the cell density and SLR factor concentration. The presence of fermentable carbohydrates in the conversion mixture increased the yield of "folic acid" 4–10 fold, and the optimum pH was 8. After conversion, practically all of the "folic acid" was contained within the bacterial cell and it could be extracted by autoclaving or by digestion with clarase.

In addition to the above series of observations in which living cells were found capable of converting an inactive growth factor for a group of microorganisms into an active form, Mims, Totter, and Day\(^\text{23}\) produced from rat liver a relatively stable enzyme preparation which was capable of producing the SLR-stimulating factor from inactive material. The enzyme was extracted by treatment with ammonium
sulfate. The proof of such conversion was based on the demonstration that the amount of SLR factor increased following incubation of the material under test with the required amount of enzyme solution at 37°C for four hours. The heat-coagulable protein was precipitated by immersing the tubes in a boiling water bath for three minutes. The supernatant liquid was then assayed for folic acid by the method of Mitchell and Snell (loc. cit.), using *S. lactis* R. as the test organism. The magnitude of increase varied from 1–5 fold for potatoes to 100 fold for some samples of yeast. There seemed to exist, therefore, a "potential folic acid factor" readily convertible by the enzyme into an active form. In more recent work, Laskowski, Mims, and Day23 have found that the enzyme has a "very generalized distribution in the organs and tissues of various animals." For example, brain, pancreas, bone, intestinal mucosa, kidney, and spleen of the rat are rich sources of the enzyme, while muscle, heart, and liver have smaller amounts. Certain tissues of the dog, hog, cow, rabbit, and chicken also possess it. The unit of enzyme was established as the amount which produces 1 γ of the SLR factor (as folic acid of potency 40,000) per hour when incubated at 37°C with 200 mgm. of yeast extract (Difco) in a total volume of 11 cc. and four hours' total incubation. Partial purification of the enzyme was achieved by adsorption, precipitation, and salting out with Na₂SO₄. An average potency of 200 units per mg. of protein was obtained in a preparation from chicken pancreas. The optimal pH was between 7 and 8.

Barton-Wright, Emery, and Robinson24 attempted to prepare folic acid from by-products of commercial liver extract manufacture. In addition to a fraction that could not be extracted from an aqueous solution by chloroform, other fractions soluble in chloroform and capable of stimulating growth of *L. casei* and *S. lactis* R. were obtained. These fractions are believed to contain three or four growth factors, and the one insoluble in chloroform is thought to be folic acid.

From the foregoing discussion of the work with the "folic acid" group of substances required for the growth especially of *L. casei* and *S. lactis* R., it is apparent that it is impossible to understand clearly at the present time the interrelationships that exist. Optimal growth of both organisms may be obtained with the same factor. With other factors either *L. casei* or *S. lactis* R. may show normal growth while the other grows to a much smaller degree. In such cases it is possible to convert by enzymatic or cellular means the inactive factor into an active form. As Luckey, Tepley, and Elvehjem25 pointed out, part of the uncertainty associated with the work would disappear if more generally accepted methods of measuring units of activity or potency were used. In an effort to improve the reliability of microbiological assays for folic acid, Luckey, Briggs, and Elvehjem26 determined the optimum amounts of biotin, nicotinic acid, pantothenic acid, pyridoxine, dipotassium phosphate, sodium acetate, tryptophan, glucose, and certain purines and pyrimidines for growth of *S. lactis* R. on a casein hydrolysate-synthetic medium. A new medium was designed on the strength of their data. Tepley and Elvehjem27 later modified this medium to allow more acid production for titrimetric determination of "folic acid." Less "scattering" of assay values when different amounts of growth are obtained was achieved in this way. Another possible source of scatter was suggested by Sherwood and Singer28 when they showed that the
grade of nonabsorbent cotton commonly used as plugs for bacteriological culture tubes contained appreciable amounts of folic acid. They used \textit{L. casei} as the test organism and found that boiling the cotton for several hours in distilled water would remove most of the folic acid.

Before concluding this section of the review another series of observations should be emphasized and extended since they are of considerable theoretical significance and may be of practical importance for assay technics. Snell and Mitchell, \textsuperscript{9} Stokstad, \textsuperscript{14} and Mitchell, Snell, and Williams\textsuperscript{15} have shown that certain pyrimidine and purine bases may be substituted for folic acid in the growth of both \textit{S. lactis R.} and \textit{L. casei}. Of the pyrimidines used, thymine (fig. 1b) seems to be the critical component, with cytosine and uracil (fig. 1c) having little activity; whereas the purines (adenine, guanine, and xanthine) are more or less interchangeable. The replacement of folic acid with thymine was also reported by Mitchell and Williams\textsuperscript{17} and by Luckey, Briggs, and Elvehjem\textsuperscript{26} in the growth of the test micro-organisms. Stokes\textsuperscript{29} found that 5000 times as much thymine as folic acid was required for maximum growth of \textit{S. lactis} and other enterococci and that a large number of pyrimidines, purines, nucleic acids, and similar compounds were inactive. Folic acid, moreover, could not be detected in cells which were grown on a medium containing thymine instead of folic acid. Stokes thus advanced the hypothesis that folic acid participates directly or indirectly as a coenzyme in the synthesis of thymine or some related compound in these micro-organisms. In testing more than 100 pyrimidines as growth factors for \textit{L. casei}, Hitchings, Falco, and Sherwood\textsuperscript{31} found 20 or more compounds of biological significance. Inhibition of growth was obtained with compounds having amino, thio, halogen, and various other substitutions in the pyrimidine molecule. These authors took exception to Stokes' hypothesis because bromouracil completely inhibited the growth of \textit{L. casei} with thymine as the nutrient but had no effect when folic acid was present. Growth does not depend therefore on thymine synthesis as such. Moreover, nitrouracil prevented growth of \textit{L. casei} with folic acid at concentrations which had little effect on growth with thymine. They suggest that the two nutrients may act as alternatives in a single system rather than as two components in the same system. Kreuger and Peterson\textsuperscript{39} emphasized the fact that thymine could completely replace vitamin B\textsubscript{9} (see below) in the nutrition of \textit{S. faecalis} and could partially replace it in the nutrition of \textit{L. casei}. In assaying material for vitamin B\textsubscript{9} by these test organisms as little as 1 gamma of thymine present free in the sample might erroneously influence the assay.

\section*{II. \textbf{Vitamin M}}

As early as 1932, Wills and Bilimoria\textsuperscript{32} showed that monkeys on a diet comparable to that consumed by certain Indian natives, largely polished rice, white bread, and "chapatti," developed anemia, leukopenia, and granulocytopenia. The bone marrow of these animals revealed a megaloblastic hyperplasia. Neither vitamin A nor C protected the animals, but the symptoms were relieved by yeast extract. Again in 1935, Wills and Stewart\textsuperscript{33} produced a dietary anemia in the rhesus monkey in which the erythrocyte count dropped from five and a half million to less than two million and the leukocyte totals went from 16,000 to less than 5,000. Both the
anemia and the leukopenia were cured with marmite (a yeast extract preparation). The same year Day, Langston, and Shukers fed young Macaca mulatta monkeys a diet believed to be adequate in protein, minerals, fatty acids, and vitamins A, B, C, and D but deficient in vitamin G (B2) and "possibly other less well known organic substances which may be essential." After a varying period on this diet the animals developed a fulminating, fatal blood disease characterized by leukopenia and anemia. Ulceration of the gums consistently accompanied the hypocythemia. Diarrhea was common. Brewer's yeast added to the diet supported good growth and prevented the deficiency syndrome. Day, Langston, and Shukers in a brief paper listed the diet fed their monkeys as consisting of whole wheat, polished rice, purified casein, Osborne-Mendel salt mixture, cod-liver oil, and oranges. The anemia and leukopenia developed and terminated fatally in 16 to 93 days. A supplement of 10 grams of dried brewer's yeast daily supported normal growth and development in the animals for 400 days, while 2.5 grams daily was inadequate. Two grams of a liver-stomach preparation daily was satisfactory.

Wills, Clutterbuck, and Evans compared the anemia induced in their monkeys by dietary means in from three months to one year to the tropical macrocytic anemia seen in Bombay. Marmite would protect against or cure this anemia as well as a fraction soluble in 80 per cent alcohol. Dried brewer's yeast was found not to be as effective as either a 0.1 per cent acetic acid extract or a 90 per cent alcohol extract. Heptoflavin was of no benefit, and wheat germ extracts were of value only when given in large doses. Day, Langston, and Darby reported the failure of nicotinic acid in doses of 10 or 50 mg. daily when used as supplements to protect monkeys from the fatal dietary cytopenia or to prolong life. This syndrome in the monkey was thus established as being different from blacktongue in dogs or pellagra in man. The factor preventing this condition was designated by these workers for the first time as "vitamin M." A more extensive report appeared in the same year (1938) from the Arkansas group when Langston, Darby, Shukers, and Day eliminated thiamin hydrochloride, riboflavin, and niacin, alone or in combination, as being responsible for the nutritional cytopenia of their monkeys. In addition to the 10 Gm. of dried brewer's yeast daily which had previously been found to protect the animals from this blood disease, 2 Gm. of a liver extract (Cohn fraction G) daily was shown to permit normal body development and maintain a normal blood picture over long periods.

As mentioned above, the vitamin M deficient monkeys were commonly found to suffer from diarrhea. This aspect of the deficiency was studied in more detail by Janota and Dack. They made blood counts and fecal bacteriological cultures before the animals were placed on the experimental diets. No dysentery organisms were found in these cultures. After one month on the deficient diet, one monkey gave positive stool cultures for bacillary dysentery and suffered from diarrhea, gingivitis, and leukopenia. By the third month seven had developed dysentery, while all of the controls remained healthy throughout this period. The following year Day, Langston, Darby, Wahlin, and Mims isolated Shigella paradysenteriae from the stools of several vitamin M deficient monkeys (fed the Goldberger diet). The deficiency syndrome was similar to that found in the earlier work of this group,
and a crude liver extract given to an animal with profound anemia and leukopenia was found to elicit a dramatic reticulocyte response and ultimate recovery. The ash of liver extract had no protective value. Niacin and riboflavin seemed to have a mild erythropoietic effect.

Wilson, Doan, Saslaw, and Schwab placed monkeys on one of three diets deficient in the vitamin B complex. Leukopenia developed in from 30 to 103 days on these diets. A period of constant leukocyte counts was usually observed before a rather sudden onset of leukopenia. All cellular elements were involved in the cytopenia that terminated fatally. Only moderate degrees of anemia were observed in some monkeys. Intramuscular injections of folic acid concentrates corrected the leukopenia in those monkeys receiving other vitamin supplements. These observations were extended by Saslaw, Schwab, Woolpert, and Wilson to show that the monkeys while suffering from the nutritional granulocytic leukopenia exhibited a lowered resistance to spontaneous infections. The mortality rate in such infections was high. Saslaw, Wilson, Doan, and Schwab then produced the vitamin M deficiency in monkeys on diets supplemented with riboflavin, thiamin hydrochloride, nicotinic acid amide, calcium pantothenate, pyridoxine hydrochloride, sodium para-aminobenzoate, choline chloride, pimelic acid, glutamine, and inositol. Only half of the animals developed significant degrees of anemia, but all developed leukopenia in from 4 to 15 weeks. Three monkeys received limited supplements of a yeast residue containing folic acid during a brief experimental period. A marked leukopoiesis and remission of clinical symptoms followed. The animals supplemented with the above vitamins plus 2 cc. of a crude liver extract did not develop the cytopenia.

Waisman, Rasmussen, Elvehjem, and Clark made a study of the nutritional requirements of the rhesus monkey employing a diet composed of sucrose, casein, salts, corn oil, and supplements of 8 members of the vitamin B complex and vitamin C. None of the animals could survive on this diet, and all showed weight loss, anorexia, moderate degrees of anemia, leukopenia, and extensive and varied lesions attributed to secondary infections with various organisms, especially those of bacillary dysentery. A biotin concentrate did not alter the course of the deficiency. Liver or liver extract prevented the deficiency. Waisman and Elvehjem then found that a norite eluate fraction of liver containing “folic acid” readily cured the vitamin M syndrome. However, they attributed part of the response to the role that folic acid might play in increasing the synthesis of biotin by intestinal bacteria. This conclusion was based on the observation that the loss of hair in the deficient monkey was not corrected by the addition of 1 per cent solubilized liver to the diet. The leukopenia was cured, but since the solubilized liver was low in biotin the return of fur in an animal was obtained only when “folic acid” was also given to the monkeys.

The anemia and leukopenia of vitamin M deficient monkeys was alleviated by daily supplements of 0.5–10 mgm. of synthetic xanomopterin according to Totter, Shukers, Kolson, Mims, and Day. A subnormal reticulocyte response of 1.5–4.5 per cent (normal, 0.2–0.4 per cent) resulted in 3 to 6 days and was maintained for 2 to 5 days. The blood cells reached normal values in 3 to 13 days and remained
normal for varying periods. It was only in the animal that received a heated liver powder in addition to the xanthopterin that the hemocytopoietic function was restored to normal for an extended period. After 71 days the blood was normal, but when the xanthopterin was withheld cytopenia promptly returned. The resumption of xanthopterin elicited a second response similar to the first. Xanthopterin alone, however, did not seem to fulfill completely the role of vitamin M, but it did delay the onset of the symptoms. Totter, Mims, and Day\(^48\) compared the folic acid content of several substances with the vitamin M activity in monkeys. The amount of folic acid present as measured by growth of \(S.\) lactis \(R.\) could account for only a small proportion of the vitamin M activity. There was much better agreement, however, between the vitamin M activity of a substance and its folic acid content if the material was first incubated with fresh rat liver before being assayed with \(S.\) lactis \(R.\). This was especially true when yeast was the substrate. The livers of two vitamin M deficient monkeys were low in preformed folic acid. Extra folic acid was produced from xanthopterin by one liver, but the other liver required yeast in addition to the xanthopterin. This latter requirement was found to prevail with chicken liver.

Day, Mims, Totter, Stokstad, Hutchings, and Sloan\(^19\) treated three monkeys made cytopenic by a deficient diet with 4 to 4.5 mg. of a highly purified preparation of \(L.\) casei factor per day for several days. There was a prompt and complete recovery. Total leukocyte and granulocyte counts had increased from normal to 7.8–10.9 per cent. Erythrocyte counts increased more slowly to normal. The infections commonly associated with this deficiency were found in one of the monkeys, and these healed following the treatment. This \(L.\) casei factor was only slightly active for \(S.\) lactis \(R.\), but treatment with the enzyme described by Mims et al.\(^22\) increased the activity for this micro-organism. Day, Mims, and Totter\(^50\) made further tests with the crystalline \(L.\) casei factor in their vitamin M deficient monkeys, using 3 mg. intramuscular injections over several days in each case. Reticulocytes rose as high as 47 per cent within 4 to 7 days in these animals. Erythrocytes, hemoglobin, and leukocytes returned to normal and the dose maintained the remission for 10 to 30 days. These blood changes were accompanied by marked clinical improvement. These authors likened this factor to vitamin B\(_6\) conjugate (see below) in its microbiological activity.

III. VITAMIN B\(_6\)

Studies in nutrition of the chick have yielded valuable information in many aspects of vitamin research. It is not surprising to find therefore that some of the early investigations with what is now commonly referred to as “folic acid” made use of this experimental animal. Stokstad and Manning\(^41\) reported that chicks on a diet adequate in all the essential elements known at the time (1938) required an additional factor for growth which could be found in yeast, middlings, alfalfa, and wheat bran. Certain stability characteristics were described. The following year, Hogan and Parrott\(^42\) published in abstract form and later in detail\(^43\) the results of experiments proving the existence of a new member of the vitamin B complex required by the chick. In the absence of this factor the animals grew slowly and de-
veloped a macrocytic hyperchromic anemia. The erythrocytes were less fragile but the coagulation time was normal. The anemia was not due to anorexia and could not be prevented by any of the known vitamins. A water soluble extract of liver was required. Because of its role in the chick, the protective substance was designated as vitamin B₁₂. Two other papers appeared in 1940 in which factors from yeast were found to be required for growth in chickens. Stokstad, Manning, and Rogers proved that a substance other than pyridoxine was required, and Schumaker, Heuser, and Norris felt that their factors R and S from yeast were probably related to those previously described by Stokstad. In neither publication was any mention made of the possibility of anemia developing, but it was stated by Schumaker et al. that no macroscopic lesions appeared.

Hutchings, Bohoyos, Hegsted, Elvehjem, and Peterson in 1941 prepared from liver a concentrate 200 times as active as the original material in promoting the growth of L. casei. They then showed that the amount of this material required for the growth of the chick was decreased in proportion to its increased activity for L. casei. They concluded that the two active principles might not be identical but that the data suggested that they were. The following year, Mills, Briggs, Elvehjem, and Hart used a basal medium which was fed day old white Leghorn chicks. These animals grew poorly and at the end of four weeks had an average hemoglobin value of 6.13 Gm. per 100 ml. blood, while chicks receiving supplements of solubilized liver extract or norite eluate factor from solubilized liver grew much better and had hemoglobin values of 8.40 and 8.09 Gm. per 100 ml. blood respectively. These experiments gave further support to the identity of vitamin B₁₂ and the L. casei factor.

O'Dell and Hogan undertook an investigation to improve the diet used for producing anemic chicks which might then be used in vitamin B₁₂ assays. A diet was found that gave an incidence of 53 per cent anemia in the animals. They suggested that higher levels of pyridoxine in the diet tended to lower the incidence of anemia because it aided in the bacterial synthesis of the antianemic principle. In support of this hypothesis was the larger percentage number of anemic animals obtained when sulfaguanidine was added to the diet. It was of interest that the eggs of hens consuming a diet rich in green foods yielded fewer anemic chicks than when the eggs came from hens fed a dry diet. Apparently vitamin B₁₂ was stored in the egg when the diet was rich in this factor. Chemical studies of the vitamin showed it to be an acid capable of forming salts with heavy metals. It could be adsorbed from acidic solution by a variety of adsorbents and was eluted with ammonia. These properties are in close agreement with those found for the various growth factors (compare above).

A brief account was published in 1943 by Pfiffner, Binkley, Bloom, Brown, Bird, Emmett, Hogan, and O'Dell in which some of the physical characteristics of a crystalline compound isolated from liver were described. This acidic substance when fed to day old chicks at a level of 2.5 γ per gram of deficient ration insured normal growth and the animals showed no anemia at the end of four weeks. It was highly active as a growth factor for L. casei since a concentration of 0.00005 γ per ml. of culture medium gave half-maximum growth of the organism. They thought
that this crystalline material was probably the same as Peterson's "eluate factor" and Williams' "folic acid." The term vitamin B₇ was retained in order to designate the pure crystalline compound they had isolated. Campbell, Brown, and Emmett in a more quantitative experiment showed that vitamin B₇ would prevent the nutritional macrocytic hyperchromic anemia in chicks at levels of 40γ per 100 Gm. of deficient ration. Growth required 100γ and a normal leukocyte level required 400γ per 100 Gm. of ration.

The ultraviolet absorption spectrum of vitamin B₇ was measured by Bloom, Vandenbelt, Binkley, O'Dell, and Pfiffner at pH values between 1.0 and 11.0 and compared with the absorption of xanthopterin at essentially the same hydrogen ion concentrations. Very striking dissimilarities were found although in general the curves were alike. Similarities were also found in curves for flavins, alloxazines and pterins.

Binkley, Bird, Bloom, Brown, Calkins, Campbell, Emmett, and Pfiffner found that yeast extracts were highly potent in vitamin B activity as measured in the anemic chick, but only 2–5 per cent of the chick antianemic activity showed in the microbiological growth effect on either L. casei or S. lactis. When this yeast concentrate was subjected to enzymatic digestion it became microbiologically active. The chemical procedure for crystallizing vitamin B was applied to the yeast digest. A crystalline compound was obtained that had the same activity for L. casei and S. lactis as the compound from liver. It also had a comparable effect on growth and on the blood picture in the chick. The crystalline compounds from liver and from "digested" yeast had identical crystallography, ultraviolet absorption spectra, and elemental chemical analyses. These observations suggested that the vitamin B activity in yeast extract was due almost entirely to a relatively simple nonprotein conjugate of the vitamin. Work with this crystalline vitamin B conjugate from yeast was continued by Pfiffner, Calkins, O'Dell, Bloom, Brown, Campbell, and Bird. The ultraviolet absorption spectrum when compared with that of crystalline vitamin B was found to differ only in values. This indicated that the two compounds had the same chromophoric groups but that the molecular size of the conjugate was 2.8 times that of vitamin B. Microbiological assay data showed that 1γ of conjugate was equivalent to 0.003–0.006γ of vitamin B, as measured by L. casei and to only 0.002γ using S. lactis R. An enzyme called vitamin B conjugase isolated from hog kidney released an amount of vitamin B from the crystalline vitamin B conjugate approximating the amount present in conjugated form as calculated from ultraviolet absorption data. The compounds from both liver and yeast gave comparable antianemic activity in the chick. From these data the authors believed their yeast compound to be different from those of Stokstad. Bird, Bloom, Emmett, and Pfiffner showed that the hog kidney extract, mentioned above, when added to the yeast vitamin B conjugate released the maximum amount of vitamin B, within approximately four hours as measured by the growth response of S. lactis R. More recently Bird, Bressler, Brown, Campbell, and Emmett described the enzymatic liberation of vitamin B from the bound form as measured with growth of L. casei. The enzyme was obtained from desiccated hog kidney and from extracts of whole almond or almond meal. The microbiological
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assays of various materials, following the enzyme treatment, were for the most part in agreement with chick antianemic assays. Campbell, McCabe, Brown, and Emmett found that 20-40 γ of crystalline vitamin B per 100 Gm. of ration would prevent the anemia, leukopenia, and thrombocytopenia in chicks during the first four weeks of life. Even 400 γ per 100 Gm. of ration did not overstimulate the production of cellular elements.

In 1944, Hutchings, Stokstad, Bohonos, and Slobodkin isolated in crystalline form a new compound from an unspecified source which was active for both \( L. casei \) and \( S. lactis \) \( R \). and was also active in the nutrition of the chick. It was 83-90 per cent as active as the compound from liver when assayed with \( L. casei \), but it was only 6 per cent as active when tested by \( S. lactis \) \( R \). assay. The new compound was required in amounts of 0.00061 γ per ml. for \( L. casei \) and 0.0042 γ per ml. for \( S. lactis \) \( R \). Absorption spectrum data made for this compound and the two previously isolated appeared to be different from Mitchell and Williams.

Two dietary factors necessary for the chick were found in liver by Briggs, Luckey, Elvehjem, and Hart. On the basis of microbiological assays these factors were distinct from folic acid. One factor was essential for proper feather development, and the other was required for growth. They were designated vitamins \( B_9 \) and \( B_{11} \). Hill, Norris, and Heuser confirmed the earlier evidence for the existence of two chick growth factors, \( R \) and \( S \), from yeast distinct from folic acid or vitamin \( B_9 \). If folic acid and vitamin \( B_9 \) are the same, a new chick antianemic factor distinct from factors \( R \) and \( S \) was thought to have been revealed. This antianemic factor might have been vitamin \( B_9 \) in the event that crystalline preparations of it were contaminated with highly potent growth and antianemic factors or stimulated the bacterial synthesis of these factors in the intestinal tract. No evidence was obtained that folic acid was required by the chick, since growth failure and mortality occurred in the absence of the antianemic factor and factor \( R \) or \( S \). Campbell, Brown, and Emmett tested the hypothesis that vitamin \( B_9 \) might alter bacterial synthesis of other vitamins in the intestine. Growth, feathering, and the blood picture of chicks were observed following oral and subcutaneous administration of the vitamin. The results for both groups were the same. Briggs, Luckey, Elvehjem, and Hart reported that vitamins \( B_9 \) and \( B_{11} \) were distinct from "folic acid" when measured by \( S. lactis \) \( R \) and \( L. casei \). Chicks fed a basal diet developed macrocytic hyperchromic anemia which was partially cured by supplements with vitamin \( B_9 \) activity. They felt that another factor was concerned with hemoglobin formation since fractions low in vitamin \( B_9 \) activity raised the hemoglobin value considerably. Fractions rich in vitamin \( B_9 \) or \( B_{11} \) did not completely prevent the anemia.

At the end of the summer of 1945 a large group of investigators reported the synthesis of a compound which was active for \( L. casei \), \( S. lactis \) \( R \), and was effective in promoting growth and hemoglobin formation in the chick. The synthetic compound was believed to be identical with the crystalline \( L. casei \) factor previously isolated from liver. This conclusion was based on several observations. Ultraviolet absorption spectra of the two compounds were identical. The refractive indices of light vibrating parallel to the length of the crystal and also to the width
of the crystals were identical for both compounds. The compounds were equally active when assayed with *L. casei* or *S. lactis* R. No data were given concerning the chemistry of this compound or its possible relationship to xanthopterin. As yet none of this information has been published.

It is clear that the work with the chick leaves several problems yet to be resolved. There is the possibility that vitamins B_{10} and B_{11} and factors R and S are related. They may represent conjugated forms of vitamin B_c which fail to reveal their potency for the chick when assayed by micro-organisms. Until this contingency is clearly eliminated, doubt must remain as to their distinction from "folic acid." There is, on the other hand, newer evidence that indicates *L. casei* factor alone is inadequate for a normal blood picture in chickens. Scott, Norris, Heuser Bruce, Coover, Bellamy, and Gunsalus reported in a note that the lactone of 2-methyl-3 hydroxy-4-hydroxymethyl-5 carboxypyridine (fig. 1d) promotes growth and prevents anemia in chicks. The use of this synthetic compound was suggested by the observation that pyridoxine previously treated with hydrogen peroxide promoted the growth of *L. casei*. Scott, Norris, Heuser, and Bruce showed that the above lactone, now designated as α pyracin, or the isomeric 4 carboxy lactone, β pyracin (fig. 1e), was required for the complete prevention of the macrocytic hyperchromic anemia that develops in chicks fed a purified diet. Of the two compounds, β pyracin was more active in promoting growth but was only slightly more active in preventing anemia. Smaller quantities of the compound prevented anemia than were required to promote growth. Hematological studies revealed that when *L. casei* factor alone was added to the diet a normocytic, hypochromic anemia developed, whereas the addition of β pyracin alone led to the development of a macrocytic normochromic type. More recently, Scott, Norris, and Heuser produced a severe hemorrhagic anemia in hens by withdrawing by cardiac puncture a volume of blood estimated as one-third the total volume. Hemoglobin regeneration was then followed daily, and it was found that injections of β pyracin and *L. casei* factor, alone or in combination, hastened the recovery process. The administration of these factors together gave higher hemoglobin values which were maintained longer than in the hens receiving only a single factor.

Briggs and Lillie observed that day old chicks fed a highly purified diet deficient in folic acid for four weeks developed characteristic deficiency symptoms such as poor growth, retarded and rough feathering, anemia, perosis, and high mortality. The survivors were then given a normal broiler ration which supported growth and feathering. After 3 to 6 weeks on this ration, however, many of the chicks developed wing and body feathers which contained large white areas and often abnormal black areas. When Wilson’s Liver Fraction L or synthetic *L. casei* factor (Lederle) was included in the diet during the first four weeks, the chicks developed normally in all respects.

The various factors which have been isolated in work on micro-organisms, monkeys, and chicks are summarized in table 1. Our knowledge of some of these factors is obviously fragmentary, and until the chemistry of synthetic *L. casei* factor becomes available it will be difficult to understand the possible interrelationships which seem to exist in these factors. Since there have been so many different sub-
ststances reported in the literature, it is felt that such a tabulation might serve as a
guide to the reader.

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Biological Activity</th>
<th>Chemical Nature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norite eluate factor</td>
<td>Liver and yeast</td>
<td>Growth factor for L. casei and S. lactis</td>
<td>Basic; related to purines</td>
<td>6</td>
</tr>
<tr>
<td>Norite eluate factor</td>
<td>Solubilized liver</td>
<td>Same as above</td>
<td>Acidic; not a nucleotide</td>
<td>7</td>
</tr>
<tr>
<td>Norite eluate factor</td>
<td>Solubilized liver</td>
<td>Same as above</td>
<td>Purine and pyrimidine present</td>
<td>8</td>
</tr>
<tr>
<td>Folic acid</td>
<td>Spinach</td>
<td>Same as above</td>
<td>Not a nucleotide</td>
<td>10</td>
</tr>
<tr>
<td>S. lactis R. factor</td>
<td>Unstated</td>
<td>Active for S. lactis. Inactive for L. casei</td>
<td>Purine and pyrimidine present</td>
<td>13</td>
</tr>
<tr>
<td>Crystalline L. casei</td>
<td>Liver and yeast</td>
<td>Liver factor active for both organisms; yeast factor half active for S. lactis</td>
<td>Methyl esters of active principle</td>
<td>14</td>
</tr>
<tr>
<td>Crystalline L. casei</td>
<td>Unstated</td>
<td>Same as above</td>
<td>Xanthopterin-like structure</td>
<td>15, 16, 17, 19</td>
</tr>
<tr>
<td>Vitamin M</td>
<td>Liver and yeast</td>
<td>Cures nutritional cytopenia in monkeys</td>
<td>Cures nutritional cytopenia in monkeys</td>
<td>37, 38</td>
</tr>
<tr>
<td>Xanthopterin</td>
<td>Synthetic</td>
<td>Partially cures cytopenia in monkeys</td>
<td>Cures trout anemia</td>
<td>46, 47</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Yeast</td>
<td>Cures chick anemia</td>
<td>Acrylic; similar to flavins, aloxozines, and pterins</td>
<td>53, 55</td>
</tr>
<tr>
<td>Factors R and S</td>
<td>Yeast</td>
<td>Required in chick nutrition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystalline Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Liver</td>
<td>Active for chick and L. casei, S. lactis</td>
<td>Acrylic; similar to flavins, aloxozines, and pterins</td>
<td>59, 61</td>
</tr>
<tr>
<td>Crystalline Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Yeast Digest</td>
<td>Same as above</td>
<td>Same as above; molecule 1.8 larger</td>
<td>62</td>
</tr>
<tr>
<td>Crystalline Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Yeast</td>
<td>Active in chick; 2-3% activity for L. casei and S. lactis</td>
<td>Same as above; molecule 1.8 larger</td>
<td>64, 65</td>
</tr>
<tr>
<td>Crystalline L. casei</td>
<td>Unspecified</td>
<td>Active for L. casei. Inactive for S. lactis</td>
<td>Absorption spectrum unlike folic acid</td>
<td>67</td>
</tr>
<tr>
<td>Vitamins B&lt;sub&gt;12&lt;/sub&gt; and B&lt;sub&gt;13&lt;/sub&gt;</td>
<td>Liver</td>
<td>Active for chick. Inactive for bacteria</td>
<td>Pyrimidine</td>
<td>29</td>
</tr>
<tr>
<td>Thymine</td>
<td>Synthetic</td>
<td>Active for S. lactis</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Crystalline L. casei</td>
<td>Synthetic</td>
<td>Active for chick, rat, monkey, bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α pyracin and β pyracin</td>
<td>Synthetic</td>
<td>Hemoglobin synthesis in the chick</td>
<td>Lactone of pyridine</td>
<td>73, 74, 75</td>
</tr>
</tbody>
</table>

IV. THE ROLE OF "FOLIC ACID" IN NUTRITION OF THE RAT

This topic has recently been reviewed by Daft and Sebrell in connection with a
more general discussion of the relationship between sulfonamides and vitamin
deficiencies. For a complete presentation of the topic, however, we feel justified in duplicating some of their effort.

The definite beginning in work of this type is sometimes difficult to ascertain. Nevertheless, in 1937 György, Goldblatt, Miller, and Fulton found that rats on a diet deficient in vitamin B₆ developed agranulocytosis, thrombocytopenia, and anemia. This pancytopenia could not be corrected by the addition of pyridoxine but required Peter's eluate. Also in 1937, Tschesche and Wolf reported that xanthopterin, as well as other pterins, would give a reticulocyte response and rise in red cell counts in rats with a goat's milk anemia. A liver preparation and also a mixture of tryptophan and histidine were also effective.

Unna, Richards, and Sampson found that rats on a diet deficient in pantothenic acid, biotin, and folic acid developed achromotrichia, retardation in growth, inflammation of the nose, and adrenal hemorrhages. The condition was prevented with pantothenic acid, but liver and rice exerted a better growth-promoting effect. Martin, on the other hand, added 1 to 2 per cent sulfaguanidine to a highly purified diet containing adequate amounts of calcium pantothenate. There was marked graying at 5 months in these animals with or without the addition of biotin. Both yeast and liver caused a sharp increase in growth rate and the graying was completely cured in two months on these supplements. When 3 mg. of a "folic acid" concentrate per day was substituted for the yeast or liver, growth gains were normal. Graying was cured completely in three rats and was only partial in the other two.

Nielson and Elvehjem studied the growth-promoting effect of folic acid and biotin in weanling rats fed 1 per cent succinylsulfathiazole. After 4 to 5 weeks on the diet, at which time a weight plateau or loss began, the vitamins were tested. Biotin alone gave very low growth responses, but when it was added to the concentrate of folic acid the definite growth responses obtained with the folic acid alone were improved. Welch and Wright and Wright and Welch added succinylsulfathiazole to highly purified diets containing all the dietary factors known for the rat. In addition to growth inhibition they observed an increase in the prothrombin time. Both deficiency symptoms were overcome by the addition of folic acid and crystalline biotin. These workers suggested that these factors promoted bacterial synthesis of other essential dietary factors in the intestine. In fact, the appearance of achromotrichia and porphyrin-caked whiskers was accompanied by a pronounced reduction in the pantothenic acid content of the liver. These signs disappeared when folic acid and biotin were added to the diet. Their role is probably secondary to that of pantothenic acid in such cases.

The development of agranulocytosis, leukopenia, and a hypocellularity of bone marrow was reported by Spicer, Daft, Sebrell, and Ashburn for rats fed either sulfaguanidine or sulfasuxidine at 1 per cent level in purified diets. Whole dried liver extracts would either prevent or cure these symptoms. Ashburn, Daft, Endicott, and Sebrell observed that these deficient animals developed hyaline sclerosis and calcification of blood vessels, necrosis of voluntary muscles, and granulocytic aplasia of the bone marrow. Certain liver fractions which were known to contain the L. casei factor when administered orally to deficient rats were found by Kornberg, Daft, and Sebrell to correct in four days the granulocytopenia and to restore
the red blood cells to normal in ten days. These recoveries were obtained during the continued ingestion by these animals of the sulfonamide-containing diet. Daft and Sebrell\textsuperscript{88} treated rats on the sulfonamide diet with 20–40\textsuperscript{g} of xanthopterin per day for four days without a response when the leukocytes had reached a level of 4,000 or less per cu. mm. and the granulocytes were 200 or less. Crystalline folic acid given orally to similar animals in doses of 20\textsuperscript{g} for four days gave an average rise of from 2,700 white cells per cu. mm. to 14,400 cells per cu. mm. The granulocytes changed at the same time from 1 to 39 per cent. Erythrocytes increased in ten days from 5.1 million to 6.9 million, and the hemoglobin rose from 9.7 to 12.8 Gm.

Axelrod, Gross, Bosse, and Swingle\textsuperscript{89} also found leukopenia and sometimes anemia in rats fed purified diets containing 1 per cent sulfaguanidin. The test substances were given orally for seven days, and both norite eluate and whole liver were effective in correcting the deficiency in leukocytes and in hemoglobin. Ran-
sone and Elvehjem\textsuperscript{90} tested the effectiveness of a folic acid concentrate in counter-
acting the decreased growth and leukopenia in rats fed sulfasuxidine in the diet and obtained approximately the same results as they did with a liver extract when each was fed at levels equal in \textit{S. lactis R.} activity. Biotin increased the growth rate when it was fed in conjunction with folic acid even when the latter was fed in insufficient quantities. Xanthopterin fed in conjunction with biotin did not increase the growth rate of the rats on the diet containing sulfasuxidine. In contrast to these observations, Totter and Day\textsuperscript{91} reported an immediate weight gain in rats on a similar diet but also containing biotin when 2\textsuperscript{g} of xanthopterin were added. They noted a pronounced leukocyte response in which the average white cell count increased from 3,420 per cu. mm. to 9,400 per cu. mm. They stated, however, that a normal distribution of white blood cells following xanthopterin therapy did not occur and that normal growth was not fully restored. It was suggested that this might be due to the injurious action of the drug as well as to the inadequacy of the xanthopterin. On the strength of the work of Totter and Day, Mitchell\textsuperscript{92} pointed out that analyses of some of his folic acid concentrates showed that xanthopterin was not infrequently present as an impurity. He recommended caution in attributing the response to folic acid alone in rats on a sulfonamide diet receiving folic acid concentrates.

Mallory, Mims, Totter, and Day\textsuperscript{93} fed yeast extracts or yeast extract concentrates to rats made leukopenic by adding succinylsulfathiazole to the diet. These substances were low in preformed \textit{S. lactis R.} stimulating factor, but the animals grew better and maintained higher total white blood cells and granulocytes than those receiving liver extract containing 1.9–15 times more preformed SLR factor. Thus there was no correlation between the effectiveness of these substances in sulfon-
amide-fed rats and their content of preformed SLR factor. But when the activity in rats was expressed as the amount of potential SLR factor present in the supplement, close agreement was obtained. These findings suggested that the factor antagonistic to the sulfonamide effect in rats was the same as or similar to vitamin M, since both could be converted into a growth stimulator for \textit{S. lactis R.}

In rats fed a highly purified diet adequate in the known required members of the vitamin B complex, Wright and Welch\textsuperscript{94} obtained a marked reduction in the
amount of folic acid and biotin stored in the liver compared with the amounts found in livers of rats fed a stock ration. The amount of these factors in liver was further reduced when succinylsulfathiazole was included in the diet. In addition there was also a reduction in the amount of pantothenic acid in the liver of these animals. The administration of crystalline biotin and a concentrate of folic acid not only caused a prompt restoration of growth but there was a recovery from the signs of pantothenic acid deficiency and a return to normal levels of the liver content of this vitamin. Welch and Wright observed that rats on a diet of powdered whole milk supplemented with minerals and the known vitamins showed no evidence of nutritional deficiency when succinylsulfathiazole was fed in amounts as large as 10 per cent. This was in sharp contrast with the usual symptoms produced at 1–2 per cent levels. The assays for folic acid in the livers of animals fed a milk diet without sulfonamide were larger than those for the livers of rats fed a highly purified diet which contained the same original amount of microbiologically active folic acid. These observations were considerably clarified in a report by Wright, Skeggs, Welch, Sprague, and Mattis in which the typical syndrome of the sulfa-induced deficiency was obtained in rats on the powdered milk diet with 10–20 per cent succinylsulfathiazole. Large amounts of folic acid were found in the feces of animals fed exclusively on powdered milk, but this elimination of folic acid was reduced by feeding the sulfonamide. Even so, a folic acid deficiency existed in rats showing considerable fecal elimination of the vitamin. By means of digestion technics it was shown that the milk contained significant amounts of "potential" folic acid which was unavailable to the micro-organisms used in assaying the diet. The rats were apparently capable of utilizing the "potential" folic acid in milk and therefore were more resistant to the sulfonamide-induced deficiency on the milk diet. Wright, Skeggs, and Welch then demonstrated that folic acid may undergo conversion in the liver of rats into materials having little or no activity as growth factors for lactic acid organisms. Evidence was obtained by showing that the folic acid content of rat liver was increased by NaCl, xanthopterin, greater dispersion of tissue, and at pH 7–8. Cyanide inhibited the yield.

Endicott, Kornberg, and Daft reinvestigated the lesions found in rats on purified diets containing one of several members of the sulfa drugs. A depletion of mature granulocytes in bone marrow with or without an increase in nucleated erythrocytes occurred. A few animals had normal marrow, and a few with normal peripheral blood had depleted marrow. Aplastic marrow was not observed in any of these rats as it was previously but there was no explanation for this difference. Hyperplasia was regularly found in rats recovering from the granulocytopenia following the administration of liver concentrates. Gross, Axelrod, and Bosse also found severe pathological changes in rats under similar experimental conditions which became maximal after two months. They had 90 per cent fatalities, which were reduced to 14 per cent by the administration of folic acid concentrate plus biotin.

Kornberg, Daft, and Sebrell observed granulocytopenia (designating less than 500 granulocytes per cu. mm. blood) in 6 of 185 weanling rats fed a purified diet without sulfonamide. All of these deficient animals showed weight loss or poor growth prior to treatment with crystalline L. casei factor, and one animal died.
The administration of the vitamin increased the polymorphonuclear cells to more
than 5000 per cu. mm. of blood and restored growth. One or more relapses occurred,
but the crystalline \textit{L. casei} factor corrected the granulocytopenia in relapse. These
important observations eliminated the possible toxic influence of the sulfonamides
in inducing the "folic acid" deficiency in rats. Kornberg, Tabor, and Sebrell\textsuperscript{16} produced
severe anemia in rats fed a purified diet containing sulfasuxidine. Anemia
was noted in only a few rats not bled but fed sulfonamide, and none showed such
severe anemia when fed a purified diet without sulfonamide even after long periods
of bleeding. Crystalline \textit{L. casei} factor was fed as a supplement to one group of rats
on the purified diet plus sulfasuxidine. The hematocrit average was 44 per cent six
days following the eleventh bleeding made during a 24 day period. Unsupplemented
litter mates under identical conditions had a hematocrit average of 19 per cent.
The average hemoglobin was 14.8 Gm. in the supplemented group and 6.6 Gm. in
the other. Of 31 rats made anemic by this procedure, 13 were treated with the \textit{L. casei}
factor. Nine showed an average hematocrit rise of from 15 per cent prior to
treatment to 28 per cent four days after treatment. The remaining untreated rats
died in an average of seven days following the last bleeding.

Higgins\textsuperscript{18} fed young male rats a purified ration supplemented with the known
vitamins. Sixteen were given 80\% of vitamin B\textsubscript{6} concentrate per day for 14 days
while receiving 50 mg. of promin daily. Twenty-nine were given the same amount
of drug and no vitamin, while 15 were given the drug and were then treated. In all
cases the administration of the vitamin had a marked antianemic effect with an
average increase in hemoglobin from 11.7 Gm. per 100 cc. to 14.2 Gm. per 100 cc.
blood. The erythrocyte volume and total red cell count showed slight increases.

A nutritional anemia in rats has recently been reported to occur after protracted
periods on a purified diet. A morphological difference distinguishes this anemia
from that due to a pantothenic acid deficiency and occurs in animals receiving this
vitamin. Carter, MacFarlane, O’Brien, Robb-Smith, and Amos\textsuperscript{19} suggest that it
may be due to folic acid deficiency.

\section*{V. FOLIC ACID IN THE NUTRITION OF OTHER ANIMALS}

As early as 1939, Wintrobe, Samter, and Lisco\textsuperscript{20} showed that weanling pigs fed
a purified diet containing yeast grew satisfactorily. If thiamine, riboflavin, and
nicotinic acid were substituted for the yeast, a severe anemia developed. When
yeast was returned to the diet, partial or complete recovery followed. Folic acid
may have been responsible at least in part for the therapeutic effect of yeast in these
experiments, but the author’s work antedated the discovery of this vitamin.

In 1942, Woolley\textsuperscript{21} fed guinea pigs a ration composed of casein, sucrose, inorganic
salts, corn oil, and vitamins A, C, D, E, K, riboflavin, pyridoxine, nicotinic
acid, pantothenic acid, choline, and inositol. The animals failed to grow and soon
died unless two new factors were added to the diet. One was soluble in 50 per cent
alcohol (GPF-1) and one was insoluble in the alcohol (GPF-2). The former was
successfully concentrated so that 5 mg. per day produced good growth. Woolley
and Sprince\textsuperscript{22} later succeeded in proving that crystalline vitamin B\textsubscript{12} was one of
the unknown factors required by the guinea pig but another as yet unidentified
factor remained.
Nielson and Black,\textsuperscript{18} using a purified basal ration, noticed that the albino mouse failed to grow after four weeks and developed a rough coat and curvature of the spine. The addition of "folic acid" to the diet supported better growth than the basal ration, but when biotin was also included normal growth and appearance resulted. In a study of spontaneous breast cancers in mice, Leuchtenberger, Leuchtenberger, Laszlo, and Lewisohn\textsuperscript{19} observed spontaneous regressions in the cancers of 38 out of 89 animals (43 per cent). The treatment consisted of daily parenteral injections of 5 γ of \textit{L. casei} factor. The incidence of new tumor development was decreased among the treated mice when compared with the controls.

A series of studies\textsuperscript{116}-\textsuperscript{119} was made in which the "B vitamin" content of various tissues was assayed during the development of rats and of chicks. The problems associated with autolyzing such tissues for assay were described and technics were recommended. The fact that folic acid was destroyed during autolysis in acid or in alkali was emphasized. It was further demonstrated that "bound" folic acid in some tissues rendered the evaluation difficult.

An interesting relationship between stilbestrol and \textit{L. casei} factor was revealed by Hertz and Sebrell.\textsuperscript{11} Normally, stilbestrol induces a marked proliferation of the tissues of the genital tract in the chick. This is reflected in an increase in weight of the genital organs and may be employed as an index of hormone activity. Chicks were maintained from hatching on a purified diet with supplements known to produce a vitamin B\textsubscript{1} deficiency; 0.5 mgm. of stilbestrol in 0.1 cc. of corn oil was given subcutaneously on each of six days. The weight of the oviducts of these chicks was consistently less than the weight of those in chicks on the same diet receiving supplements of \textit{L. casei} factor. The weight difference was obtained whether the \textit{L. casei} factor was administered from hatching or only as a curative measure during the last ten days preceding autopsy. Since the response to stilbestrol was unimpaired in pantothenic acid deficient chicks, debility limitations of growth were not sufficient to affect a reduction in oviduct response observed in the "folic acid" deficient chicks. These data indicated that an adequate intake of \textit{L. casei} factor was essential for normal metabolism of stilbestrol in these animals.

Krehl and Elvehjem\textsuperscript{115} placed young dogs on a synthetic ration low in nicotinic acid. A severe deficiency resulted. There was a poor response to added niacin which was soon followed by a relapse and death despite the administration of this vitamin. When the basal ration was supplemented with a "folic acid" concentrate, a nicotinic acid deficiency was obtained which responded adequately and consistently to nicotinic acid.

In the field of invertebrate nutrition, Tatum\textsuperscript{116} showed that the larvae of \textit{Drosophila melanogaster} grown under sterile conditions required a water-soluble yeast extract and an insoluble residue of yeast autolysate for normal growth. The basal agar medium contained amino acids, carbohydrate, salts, and thiamine, riboflavin, pyriodoxine, nicotinic acid, and calcium pantothenate. More recently, Golberg, De Meillon, and Lavoipierre\textsuperscript{117} in studies of growth factors required for the larvae of \textit{Aedes aegypti} \textit{L.} found that folic acid was necessary for pupation. Its effect could not be replaced by xanthopterin or thymine. Folic acid also exerted an important effect on growth and survival rates, body pigmentation, and size of the larvae. Transference of the larvae from a folic acid-free medium to one containing the
vitamin, and vice versa, revealed that the most vital effect seemed to occur during the third stage of larval life. Kidder obtained quantitative data to prove that the increase in population of his ciliate, Tetrohyphlena geleii W, grown aseptically, was in direct proportion to the concentration of Williams' folic acid of potency 5000. This preparation gave half-maximal growth at levels of 0.002 μg per ml. of medium.

VI. STUDIES OF "FOLIC ACID" IN HUMAN SUBJECTS

It is impossible in a review of this length to make a complete or comprehensive report on all of the earlier work in which substances were used in the treatment of anemia that might now be recognized as containing "folic acid." To make the attempt would require that the brilliant demonstrations of the therapeutic effect of liver in pernicious anemia be included. Since liver therapy is so universally employed in medical practice, this aspect of the work is being omitted. We shall attempt, however, to give a background of thought and clinical experimentation which, combined with the evidence presented above, made the testing of L. casei factor in human beings seem an obvious step.

In 1931, Wills demonstrated the curative effect of a yeast extract in macrocytic anemia of pregnancy which occurs commonly in India. Some years later Wills and Evans found cases of tropical macrocytic anemia which did not respond to the more highly purified liver extracts which were of therapeutic value in cases of pernicious anemia. This suggested the existence of a new hemopoietic factor in crude liver and autozoyte yeast extracts. This new factor could not be identified with thiamine, lactoflavin, or nicotinic acid but was present in a yeast-fuller's earth filtrate. Wintrobe reported maximal hemopoietic responses in five patients with pernicious anemia given dehydrated brewer's yeast at a level of 1-2 Gm. per Kg. body weight per day. In one case oral liver extract was more effective than the brewer's yeast. Nine patients on a maintenance level of 0.3-0.8 Gm. per Kg. body weight per day remained well for 4 to 10 months. Reticulocytosis occurred in two patients given yeast extracts parenterally but a significant increase in number of erythrocytes did not follow.

Several reports have appeared during the past few years concerning the anemia of pregnancy. Miller and Studdert made a study of 23 cases in which all were found to respond to specific therapy. Treatment could be discontinued in 14 of these cases. Dietary deficiencies and vomiting were important factors associated with the etiology of the anemia. Free hydrochloric acid was present in the gastric juice of 18 cases. In these, marmite plus a good mixed diet was usually sufficient treatment. In the remaining cases, where no free acid was present, or in those patients not responding to marmite, remission of the anemia followed injections of either a refined or crude liver extract. A degree of iron deficiency often became apparent during therapy. Davidson, Davis, and Innes reported on 16 cases of anemia occurring during pregnancy which resembled Addisonian pernicious anemia. A megaloblastic bone marrow was found. Perseverance and intensive therapy in refractory cases was considered to be of vital importance. In three cases of macrocytic anemia of pregnancy and the puerperium, Fullerton concluded that a deficiency of the antipernicious anemia principle was not the only factor concerned
in the production of the anemia. Reticulocytosis following injections of liver extracts was not always followed by erythrocyte regeneration. Whole liver appeared to provide other factors necessary for restoring the blood to normal. He considered his cases analogous to the anemia associated with steatorrhea (see reference 138).

Anemia associated with deficiencies of the vitamin B complex has been studied by Moore, Minnich, Vilter, and Spies. Among 30 patients with hypochromic anemia, 32 had a clinical vitamin deficiency. All were found to respond satisfactorily to oral administration of ferrous salts. Brewer's yeast administered in some cases in doses of 25 Gm. three times daily did not alter the rate of increase of hemoglobin when compared to cases receiving iron but no yeast. Moore, Vilter, Minnich, and Spies later reported that the oral or parenteral administration of a combination of niacin, thiamine, riboflavin, calcium pantothenate, pyridoxine, inositol, para- amino-benzoic acid, and choline had no effect in correcting the macrocytic anemia in patients with pellagra or deficiency of the vitamin B complex. This work was done on 25 patients with red blood cell counts under three million, whose diets had been deficient in animal protein and B vitamins for years. Clinical signs of vitamin deficiency such as glossitis, cheilosis, or peripheral neuritis were usually found. Nine of 10 patients gave a reticulocyte response to daily injections of 4-8 U.S.P. antipernicious anemia units in the form of highly purified liver extracts. Eight of these showed a marked acceleration in rate of increase in erythrocytes. A reticulocyte response followed the feeding of beef muscle and an 80 per cent alcoholic extract of beef in other cases. They felt that the anemia was due to a prolonged dietary deficiency of extrinsic factor often associated with poor absorption from the intestinal tract and possibly inadequate production of intrinsic factor by the gastric mucosa. Watson and Castle reported the results of studies on three cases of nutritional anemia. All gave histories of dietary inadequacies, all had free hydrochloric acid, and two of the cases became anemic during pregnancy. The blood showed a macrocytic hyperchromic anemia with mild leukopenia and thrombocytopenia. Anisocytosis and poikilocytosis were less marked than in comparably severe pernicious anemia. The differential white cell count was normal. There were no neural manifestations. They found a prompt response to orally administered liver extracts immediately following therapeutic failure of liver extracts given parenterally, even in multiple U.S.P. units daily, in two cases. This showed a deficiency of some substance other than that effective in pernicious anemia. The third case indicated that intramuscular injections as a route for therapy were unsatisfactory in that ten times the normal amount was required.

A study was undertaken by Castle, Ross, Davidson, Burchenal, Fox, and Ham to test additively the extrinsic factor activity of all members of the vitamin B complex and of certain other accessory nutritional factors. Casein was rendered free of extrinsic factor and administered simultaneously in the belief that one or more of the vitamins might become active as a prosthetic group on the casein molecule as a result of the action of gastric juice. They observed that the procedure required to convert crude casein into a "vitamin free" form was also essential for the elimination of the extrinsic factor and that extrinsic factor was not recon-
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stituted when the vitamins were combined with the casein so treated, under the conditions and tests of this experiment. It was concluded, nevertheless, that extrinsic factor should still be regarded as an as yet unidentified thermostable component of the vitamin B complex.

Wright and Welch\textsuperscript{129, 130} measured by the \textit{L. casei} growth method of Landy and Dickens\textsuperscript{12} the daily urinary excretion of folic acid in 15 normal human subjects. The average value obtained from 42 samples was 0.0108 mgm. units. The average daily intake of folic acid in well fed adults is 1.4 mgm. units. This figure is derived from a report by Williams\textsuperscript{131} in which he states that a daily intake of 0.1 mgm. unit of folic acid is sufficient and the average figure was based on an assay of a well rounded mixed diet. When the urine samples were subjected to autoclaving with dilute acid or dilute alkali, or to digestion with takadiastase, no increase in the amount of folic acid was found. Incubation of the urine with a fresh rat liver preparation caused the appearance of more folic acid. The yield of the substance in urine which seems to be converted into the vitamin was increased by autoclaving for one and one-half hours in the presence of normal HCl. This treatment destroyed the free folic acid present. Since synthetic xanthopterin could be converted into folic acid under similar conditions, it was thought that possibly uropterin, said to be identical with xanthopterin, was the substance in urine capable of being enzymatically converted into folic acid. This reasoning would suggest that xanthopterin may constitute one part of the vitamin molecule. Johnson, Hamilton, and Mitchell\textsuperscript{132} verified the low urinary excretion of folic acid in normal human subjects reported by Wright and Welch. They found, however, that sweat contained five times as much folic acid as the urine when assayed with \textit{L. casei} and about six times as much when \textit{S. lactis} was the test organism. These values were obtained under conditions of profuse sweating and were on an hourly basis. They failed to increase the folic acid content of urine by incubation with vitamin B conjugase or takadiastase. The high ratio of excretion of folic acid in sweat when compared to urinary excretion is contrary to the values found with other members of the vitamin B complex.

In 1944, Sharp, Vonder Heide, and Wolters\textsuperscript{133} reported the results of preliminary clinical studies on the antianemic action of vitamin B\textsubscript{0} in the form of a yeast concentrate. Ten patients, all of whom had been under observation for a year or more, were known to give no response to various types of antianemia therapy tested during this period. All had erythrocyte counts between 3.0 and 3.5 millions per cu. mm. and 9–10 Gm. of hemoglobin per 100 cc. of blood. The vitamin B\textsubscript{0} concentrate was administered in amounts giving 600 γ per day, and after the first week it was increased to 1500 γ per day. At the end of four weeks' treatment the hematocrit showed an appreciable increase, but otherwise there was little change in the blood picture.

Because of the results obtained with "folic acid" in the treatment of vitamin M deficiency in monkeys, it was considered worth while to test the effect of this substance on the leukopenia commonly seen in persons with a multiple vitamin B complex deficiency. A preliminary report by Berry, Spies, and Doan\textsuperscript{134} indicated that either a "folic acid" concentrate from liver or the newly synthesized\textsuperscript{12} \textit{L.
casei factor when administered parenterally might elevate the total number of circulating leukocytes with a proportionate increase in granulocytes in some cases. There was a left shift in the Arneth nuclear index accompanying the rise, but the elevation was relatively transitory and in most cases was not maintained the following day. Of interest in this connection was a recent report by Watson, Sebrell, McKelvey, Daft, and Hawkinson in which elevations of leukocyte counts were obtained in each of 6 cases of leukopenia resulting from Roentgen ray therapy following oral administration of L. casei factor concentrate. Five of the 6 cases were being irradiated for carcinoma of the cervix and the other for polycythemia vera. No effect was observed in a case of severe leukopenia following x-ray therapy for Hodgkins’ disease nor in 8 cases of refractory anemia.

Spies, Vilter, Koch, and Caldwell also reported that a crude folic acid concentrate yielded no hematological response along with niacin, thiamine, riboflavin, calcium pantothenate, pyridoxine, inositol, para-amino-benzoic acid, choline, pyridoxamine, and pyridoxal in treating macrocytic anemia. They reported that materials of unknown structure isolated by Cline from reticulogen, given parenterally to patients with macrocytic anemia, had produced reticulocyte responses of varying degree. However, in some cases there was no positive evidence of erythrocyte regeneration with 10–12 per cent reticulocytes, and in other cases a brief and minimal rise in reticulocytes was followed by an increase in erythrocytes, leukocytes, and hemoglobin. As Houghton and Doan had previously emphasized, reticulocytosis alone is an insufficient index of erythrocyte maturation in the bio-assay of potential hematopoietic substances. Peripheral blood counts, bone marrow differential counts, and plasma iron value may all be required. Spies et al. tested synthetic L. casei factor on 5 cases of macrocytic anemia in relapse. The material was dissolved in saline made slightly alkaline with small amounts of NaHCO₃ and given parenterally. There was reticulocytosis and a slight increase in erythrocytes and hemoglobin in each case. Four additional macrocytic anemias were administered oral doses of L. casei factor. All responded in a way comparable to that obtained following intravenous injection of the vitamin. The dosage ranged from 50 mgm. twice a day to 50 mgm. three times a day. A later report by Vilter, Spies, and Koch was based on the study of 14 cases of macrocytic anemia. Six of these cases were diagnosed as nutritional macrocytic anemia, 5 cases were of Addisonian pernicious anemia, and 3 cases were of indeterminate types. All but 2 of the patients were hospitalized and their diets were closely regulated so that sources of extrinsic factor would be minimal. Only 1 patient received other vitamins concomitantly with the synthetic folic acid of the Lederle Laboratories. Thirteen of the 14 patients showed a positive hematological response consisting of reticulocytosis and subsequent rise in erythrocytes, hemoglobin, and leukocytes. In those cases in which treatment was continued the regeneration continued to normal levels (figs. 2 and 3). Erythrogenesis occurred regardless of the route of administration of the folic acid and regardless of the clinical classification of the macrocytic anemia. The responses were considered to parallel those afforded by liver extract.

Moore, Bierbaum, Welch, and Wright also obtained clinical and hematologic
remissions in 2 patients with Addisonian pernicious anemia following the administration of synthetic *L. casei* factor. One of these patients received a daily oral dose of 100 mg. for 10 days. The initial red cell count rose from 1.2 million to 3.0 million beginning about the seventh day of therapy and was constant for four
weeks. There were 40 per cent reticulocytes on the seventh day. The second patient started at 0.7 to 0.95 million, was transfused to 1.4 to 1.5 million, following which an oral dose of 30 mg. folic acid daily for 14 days was given. There were 44.5 per cent reticulocytes on the eighth day and the red cells began to rise on the sixth, reaching 2.5 million on the fourteenth day. The leukopenia and thrombocytopenia were corrected. One case of nontropical sprue was given 20 mg. L. casei factor per day for 10 days and 40 mgm. every two days, all parenterally. The initial erythrocyte count of 2.6 million rose to 3.5 million and leveled off after 12 to 14 days. The maximum reticulocyte response of 30.2 per cent occurred on the seventh day. Leukocytes and platelets were normal within 7 to 10 days. A case of pernicious anemia of pregnancy was given 20 mgm. folic acid parenterally daily for 10 days. The erythrocytes increased from 1.1 million to 3.0 million in 15 days and 48.2 per cent reticulocytes were found on the seventh day.

A preliminary report by Darby and Jones on the treatment of nontropical sprue with synthetic L. casei factor appeared late in 1945. One case was followed for fifteen days and the second for four days. Reticulocytosis occurred in both cases and the former showed an elevation in total number of erythrocytes. More recently, Darby, Jones, and Johnson have reported the treatment of three cases of sprue, all fulfilling the diagnostic criteria set up by Hanes for this disease, with 15 mgm. of synthetic L. casei factor daily by the intramuscular route. The glossitis disappeared in 3 to 4 days followed by rapid regeneration of papillae. There was an improved sense of well-being, the diarrhea subsided, the appetite improved, and there was a decided gain in body weight. The maximum reticulocyte response was 15.3 per cent on the eighth day in one case, 43 per cent on the sixth day in another, and the third case had 11 per cent on the sixth day (day of publication). There was an increase in number of erythrocytes, grams of hemoglobin, platelets, and leukocytes. The sternal marrow returned to normal. At essentially the same time, Spies, Lopez, Menendez, Minnich, and Koch reported from Cuba the preliminary results of oral administration of 100 mg. synthetic folic acid twice daily to three selected cases of tropical sprue. The red blood cells ranged from 1.15 millions to 2.16 millions; the hemoglobin from 6.6 to 9.5 Gm., and after 10 days of therapy there was an elevation in both erythrocytes and hemoglobin. The maximum reticulocyte responses were 17.2, 17.2, and 22.7 per cent for these cases.

More extensive reports by Spies definitely establish the therapeutic value of synthetic L. casei factor in various clinical types of macrocytic anemia. Remissions were obtained in 5 cases of nutritional macrocytic anemia; 5 with Addisonian pernicious anemia; 8 with sprue (fig. 4); 3 macrocytic anemias of pregnancy; 1 macrocytic anemia associated with chronic alcoholism, cirrhosis of the liver, and neuritis; 1 macrocytic anemia associated with carcinoma of the stomach; and 3 of indeterminate etiology. No response was obtained in 3 cases of aplastic anemia, 3 cases of leukemia, and 4 cases of iron deficiency anemia. Dosages as large as 400 mg. by mouth were given without ill effects. Five cases responded to 10 mg. per day for ten days and all had previously failed to respond to doses of 5 mg. per day for 10 days.

Spies, Milanes, Menendez, Koch, and Minnich extended the earlier observa-
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Recent observations on the therapeutic value of folic acid in tropical sprue. They emphasized the fact that folic acid should be considered an antianemic substance and therefore is not necessarily identical with the antianemic substance(s) present in liver extract or dried brewer's yeast powder. In treating these cases of sprue the patients improved both clinically and hematologically while restricted to a diet devoid of meat and meat products. The authors do not recommend such a restriction in diet under normal conditions of treatment but rather stress the importance of a high vitamin and high protein diet in hastening recovery and convalescence. Additional unpublished observations by Spies and his associates in Cuba and Puerto Rico indicate that folic acid has a profound effect on the alimentary tract of persons with sprue. Roentgenograms of the gastro-intestinal tract show that the highly irritated small bowel tends to become normal following *L. casei* factor therapy.

![Graph showing the effect of folic acid therapy on hemoglobin, red blood cells, and reticulocytes over a period of time.](image)

**Figure 4. Case of Sprue—Folic Acid Therapy**

There is also 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 (fig. 5). It has been found, however, that thiamine and niacin deficiencies may also contribute to the disorder. Frequently the red tongue of sprue can be relieved promptly by the administration of niacin alone. Folic acid has been found to produce a hemopoietic response in cases of tropical sprue simultaneously infected with intestinal parasites and even in the presence of severe pneumonia. These cases did not receive any specific treatment for the infections and hence responded to folic acid despite them.

Clinical evidence for the hemopoietic value of synthetic folic acid is rapidly accumulating: Moore, Bierbaum, Heinle, and Welch,113 Goldsmith,114 Doan, Wilson, and Wright,115 and Vilter, Vilter, and Spies.116 All of these investigators have obtained remissions in all cases of macrocytic anemia. The smallest dosage level so far reported is that of Doan et al., who obtained a complete remission in a case of Addisonian pernicious anemia with 2 mg. of folic acid given parenterally.
FIGURE 5. STOOL SAMPLES FROM A CASE OF TROPICAL SPRUE (a) BEFORE TREATMENT AND (b) FOLLOWING TREATMENT WITH SYNTHETIC L. casei FACTOR
daily for 20 days. The ability of this substance to maintain a normal blood picture is being investigated in various clinics. Until sufficient time has elapsed for more thorough testing, some doubt must remain as to the completeness with which folic acid can be expected to replace the standard liver therapy employed in such cases. However, Doan et al. and Spies have independently recommended folic acid for patients sensitive to liver extract and suggest that this is a safe and satisfactory procedure in such cases. Doan et al. also tested without success the value of folic acid in correcting the leukopenia of influenza and the panhematopenia of myelophthisic and idiopathic states.

The administration of the synthetic *L. casei* factor to normal individuals has been found to produce no effect on the blood picture. Unpublished studies on 18 college students and faculty members selected at random have been conducted over a period of several months. Each person was given 50 mgm. folic acid daily for two weeks. No change in the blood was observed except in those cases showing slight red cell deficiency. In every case the blood count was normal or slightly elevated at the end of administration. In 4 subjects the daily dose was continued for two to three months and the blood picture remained unchanged. These observations, along with others, suggest that folic acid has no undesirable action under the conditions of these studies.

These recent developments, therefore, offer bright hope for the long-sought clue to the riddle of macrocytic anemia. Before too optimistic a view is taken, however, it might be wise to reflect on some of the questions which must be resolved concerning the place *L. casei* factor occupies in this problem. As Moore et al. and Spies et al. point out, complete remissions have not as yet been obtained with the quantity of folic acid which Clark has recently shown to be present in therapeutic doses of liver extract. Moreover, SubbaRow, Hastings, and Elkins in a recent review state that Loland, Klem, Strandell, and associates in Sweden have obtained hemopoietic responses with 0.7 mgm. of a liver fraction. From the description of the properties of this fraction, it seems unlikely that it is identical with (or even related to) the *L. casei* factor. SubbaRow et al. also, on the basis of their own experimentation, lean toward the view that there is no single antianemic principle in liver but rather that an interaction of as many as three different substances may be required. All of these factors may act in such small amounts that the chance for contamination seems unlikely. In any event, it seems safe to conclude that *L. casei* factor is not extrinsic factor since it acts orally in the absence of normal gastric juice and is also effective parenterally.

Recent observations by Spies, Vilter, Cline, and Frommeyer add another contribution to the knowledge of antianemic substances. In one case of macrocytic hyperchromic anemia in relapse, with persistent histamine refractory achylia and achlorhydria, 2 Gm. of thymine were given orally thrice daily for 14 days. A peak reticulocytosis of 14.2 per cent was obtained on the eleventh day of treatment, and on the twentieth day after treatment the erythrocytes had increased from 1.01 millions to 2.74 millions and the hemoglobin had risen from 6.3 Gm. to 9.4 Gm. The bone marrow reverted from a megaloblastic to a normoblastic state. The same patient had previously failed to respond to 500 mgm. of thymine orally twice daily for 6 days. Spies, Frommeyer, Vilter, and English have since reported remissions...
following large doses of thymine in three cases of Addisonian pernicious anemia in relapse. These patients were hospitalized and were given a diet devoid of meat or meat products. Doses up to 3.4 Gm. three times daily for 11 days were used. The clinical and hematological improvement was found to be similar to that which follows the administration of folic acid to patients with pernicious anemia in relapse.

Since thymine is part of the nucleic acid molecule, and since nucleic acids have long been known to play an important role in cellular metabolism, there is the suggestion that antianemic factors are linked to nucleic acid synthesis in some way. There seems to be little doubt but that liver extracts contain antianemic principles that are not identical with folic acid and could not possibly contain sufficient thymine. Thus Castle's "erythrocyte maturation factor" must either be the end product of the action of these several substances or else be several different substances capable of leading to the same end result. Should this end result be bound in with nucleic acid formation, it would seem, a priori, that folic acid and other antianemic factors may act in a similar over-all manner but possibly by different routes. Until more specific data are accumulated regarding the biochemistry of these compounds it would doubtless be wiser to omit further unfounded speculation. There is, however, every indication that the practicing physician will soon recognize in folic acid or thymine therapeutic tools of tremendous value in the handling of cases of macrocytic anemia in relapse. While the effect of thymine is of great scientific interest, it is of little practical importance since the dosage required is so very large, being somewhere in the neighborhood of 1200 times by weight the amount of folic acid required to produce a similar response.

Spies and associates have included pernicious anemia in the group of macrocytic anemias that respond to folic acid, despite the fact that they realize that its natural pathogenesis is somewhat different from the macrocytic anemia of sprue, pregnancy, pellagra, and from nutritional macrocytic anemia. Spies has considered that folic acid works as a part of an enzyme system and that it could be built up in the body and thus be made into an antipernicious anemia factor. His working hypothesis, however, is that folic acid in food occurs as a conjugate and that it is likely that in pernicious anemia the enzymes are unable to liberate efficiently the folic acid, whereas in persons with sprue, pellagra, pregnancy, and nutritional macrocytic anemia this substance and substances that act similarly are made more available. He and Moore have consistently stressed that a satisfactory explanation has not been made concerning the fact that a large amount of folic acid is required to produce a satisfactory hemopoietic response in contrast to a highly potent liver extract in which the active substance is smaller. This naturally leads to the thought that perhaps the folic acid conjugates are stored in our bodies and that liver extract may contain a substance capable of liberating from them the substance which acts on the bone marrow.

Today at least four crystalline compounds have been obtained from liver, yeast, and other sources. These compounds have somewhat different properties, and soon their relationship one to the other will be greatly clarified, since the chemical structure is becoming known.
The liver *L. casei* factor has been isolated and synthesized, and the formula, which is as follows, has recently been announced:

\[
\text{COOH} \quad \text{O} \quad \text{N} \quad \text{N} \quad \text{NH} \quad \text{C} \quad \text{N} \quad \text{N} \quad \text{NH} \quad \text{CH} \quad \text{O} \\
\text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{N} \cdot \text{[4-[(2-Amino-4-hydroxy-6-pteridyl) methyl]amino benzoyl]glutamic Acid}}
\]

The fermentation *L. casei* factor differs in the number of molecules of glutamic acid, as they both contain the same heterocyclic ring structure. Perhaps the term "pteroylglutamic acid" will be a suitable chemical name for the liver *L. casei* factor.

Spies, in unpublished observations, has had cases of Addisonian pernicious anemia, nutritional macrocytic anemia, and sprue which have given complete remission and have developed normal blood values. In those cases which did not develop entirely normal blood values, synthetic vitamins and liver extract were tried independently and together and in no instance produced any augmentation.

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