PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS

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It is a common observation that the interpretation of scientific studies often becomes possible in the light of knowledge that is subsequently acquired. This observation has a strong application to an evaluation of the early work in the field of nutritional deficiencies. Descriptions of the specific lesions arising from the lack of a vitamin have commonly preceded alike the experimental definition of the uncomplicated deficiency and the chemical understanding of the missing nutrient. Particularly is this to be noted in the case of the vitamin B factors. The recognition and identification of the “newer” members of the vitamin B complex was preceded by early work which described nutritional lesions. With the progress of knowledge, more complete basal diets became possible as one by one the B vitamins were made available in pure form. In the case of pteroylglutamic acid, the interpretation of biological results was complicated by the existence of several naturally occurring derivatives of a parent molecule and by the variation which was encountered among different species of test organisms in response to these derivatives.

The biological effects of pteroylglutamic acid have been observed under a variety of circumstances which have given rise to a number of different names for the vitamin. The more commonly used names are listed in table 1. The name “folic acid” is widely used, doubtless due to its brevity and euphony, but since this name refers to an unidentified factor which was measured with Streptococcus lactis R, it is preferable to adopt the somewhat more cumbersome but chemically defined term, pteroylglutamic acid. Not included in table 1 are two conjugates which have been isolated in pure form; “fermentation L. casei factor” and “vitamin B conjugate”. For “fermentation L. casei factor” (1) the name pteroyltriglutamic acid has been used (2), and in keeping with this nomenclature we have used the term pteroylheptaglutamic acid in preference to the longer name pteroylhexaglutamylglutamic acid (3) for “vitamin B conjugate”.

The complexity of the nutritional relationships of the pteroylglutamic acid “family” of compounds has been to some extent lessened by recent studies in the fields of enzymology and structural chemistry. The following observations bear on the subject.

1. The parent molecule, pteroylglutamic acid, consists of a pteridine grouping linked through para-aminobenzoic acid to a single glutamic acid residue (fig. 1).

2. Successive molecules of glutamic acid may be attached in peptide linkage to the first glutamic acid radicle. Compounds with respectively three and seven glutamic acid groups have been isolated. These compounds have been termed “conjugates”.
3. The growth of the test organism *S. fecalis* R is not markedly promoted by either of the two conjugates; the conjugate with seven glutamic acid groupings does not appreciably stimulate the growth of *Lactobacillus casei*.

4. Certain animals, including chicks, rats and, probably, monkeys, can utilize the conjugates as sources of pteroylglutamic acid when fed.

5. The conjugates are split with the liberation of pteroylglutamic acid by specific enzyme systems which are present in mammalian liver and kidney, avian pancreas, and other animal tissues. Such a system is also present in

| TABLE 1 |
|---|---|---|---|
| **NAME** | **DESCRIPTION** | **DATE** | **REFERENCE** |
| None | Yeast extract, effective in the treatment of tropical macrocytic anemia | 1931 | (4) |
| Vitamin M | Yeast and liver extract, effective against nutritional cytopenia in monkeys | 1938 | (5) |
| Factor U | Yeast extract, promoted growth in chicks | 1938 | (6) |
| Vitamin B₉ | Adsorbed on fuller's earth, prevented nutritional anemia in chicks | 1939 | (7) |
| Norite eluate factor | From yeast and liver, active for *L. casei* | 1940 | (8) |
| Folic acid | Active for *S. lactis* R; concentrates prepared from spinach | 1941 | (9) |
| *L. casei* factor | Isolated from liver and yeast | 1943 | (10) |

In addition to the above, less clearly defined fractions have received separate names, including vitamins B₁₀ and B₁₁ (11) and factors R and S (12).

certain vegetable materials, including almonds and potatoes, but is perhaps absent from *S. fecalis* R. These observations are thought to account for the finding that certain preparations were active when fed to deficient animals but were relatively inert in the microbiological assay. Treatment of such preparations with the specific enzyme system usually increases their potency in the microbiological assay.

6. The erythrocyte maturation factor of U.S.P. concentrated liver extracts is not identical with pteroylglutamic acid, although the factor and pteroylglutamic acid both produce identical hemopoietic responses in patients with addisonian pernicious anemia.

7. An "incomplete" molecule, pteroic acid, has been synthesized by omitting glutamic acid. It consists of a pteridine grouping linked to para-aminobenzoic
acid, and is biologically active for *S. fecalis R* but not for *L. casei* or for chicks or rats (table 2).

*Tropical macrocytic anemia and its counterpart in monkeys.* A description of "tropical macrocytic anemia" appeared in 1931 (4). The disease was found to have a blood picture similar to that in addisonian pernicious anemia but the other symptom-complexes associated with pernicious anemia were not present. The condition was observed in women patients in Bombay and was at times complicated by pregnancy. The administration of "Marmite", a concentrated extract of autolyzed yeast, 4 grams 2 to 4 times daily, was found to relieve the anemia. In a further communication (13) a similar condition was described in monkeys, the use of which animals had been undertaken to investigate the nature of the missing dietary factor needed in tropical macrocytic anemia. The animals were placed on a diet similar to that consumed by women in Bombay who developed the anemia and a macrocytic anemia was produced in the monkeys which could be cured by feeding Marmite or by oral or parenteral administration of liver extracts which were active in the cure of tropical macrocytic anemia in human patients.

Studies of the bone marrow in the anemic monkeys showed a preponderance of megaloblastic cell types, and photographs of bone marrow preparations indicated a contrast between the marrow pictures respectively in monkeys with the nutritional anemia, in normal monkeys and in monkeys rendered anemic by massive hemorrhage (13). Further experiments (14) were concerned with studies of the active factor in liver; the erythrocyte and reticulocyte responses in de-

![Fig. 1. Pteroylglutamic acid; N-\(\text{4-[(2-amino-4-hydroxy-6-pteridyl) methyl]amino}\)-benzoyl] glutamic acid](image)

**TABLE 2**

<table>
<thead>
<tr>
<th>NAME</th>
<th>RELATIVE ACTIVITY</th>
<th>ACTIVITY FOR</th>
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<tr>
<td></td>
<td><em>S. Fecalis R</em></td>
<td><em>L. Casei</em></td>
</tr>
<tr>
<td>Pteric acid</td>
<td>50</td>
<td>0.01</td>
</tr>
<tr>
<td>Pteroylglutamic acid</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Pteroyltriglutamic acid (Fermentation <em>L. casei</em> factor)</td>
<td>7.5</td>
<td>80</td>
</tr>
<tr>
<td>Pteroylheptaglutamic acid (Vitamin B$_6$ conjugate)</td>
<td>0.3</td>
<td>0.2</td>
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ficient monkeys were used as criteria in the assay. It was possible to differentiate the factor from the anti-pernicious-anemia factor of concentrated liver extracts; two preparations were encountered which were active for pernicious anemia in human subjects yet which produced no response in the experimental monkeys. In another article (15), the fractionation of liver extract by treatment with saturated ammonium sulfate was described; it was found that the anti-pernicious-anemia factor was precipitated, while the factor effective in monkey anemia was not precipitated. An extract prepared from yeast was subjected to similar treatment, and again the active factor for monkeys remained in solution. The factor was differentiated from thiamine, riboflavin and nicotinic acid (14).

The above observation that several types of purified liver extract which were potent in the treatment of pernicious anemia had no effect in curing the anemia in monkeys even when administered in "enormous doses" was paralleled by similar observations with human subjects with tropical macrocytic anemia. It was found that seven cases did not respond to a batch of "Anahaemin" which had been demonstrated to be effective in the treatment of pernicious anemia (16). There was no reticulocytosis, no clinical improvement, and no appreciable rise in red cell count during the 10-day period of treatment. Anahaemin has been described (17) as "a concentrated preparation of the erythrocyte maturation factor prepared by the method of Dakin and West. During precipitation it is completely precipitated with 99 per cent alcohol, taken through two ammonium sulfate fractionations, and one reineckate precipitate. Anahaemin in 2 cc. contains a fraction derived from 450 grams of fresh liver".

It has been postulated that the factor of Wills differs from both pteroylglutamic acid and the erythrocyte maturation factor (18). The evidence for the differentiation from pteroylglutamic acid was not striking; a patient failed to respond to the very small dose of 1.3 milligrams of pteroyltriglutamic acid for 10 days, following which a response was obtained to large doses of a crude liver preparation. It has been established (19, 20) that pteroylglutamic acid will cure a deficiency symptom in monkeys which appears to be similar in all respects to the condition first described by Wills and co-workers and it is also evident that pteroylglutamic acid will produce a prompt remission in nutritional macrocytic anemia and in the macrocytic anemia of pregnancy (21, 22, 23), indeed there is one report (24) describing remission of macrocytic anemia of pregnancy following treatment with pteroylglutamic acid in India under conditions which would appear to relate to the earlier observations (4). The pteroylglutamic acid content of Marmite is not known, but yeast, from which Marmite is prepared, is a good dietary source of pteroylglutamic acid and its conjugates. The preponderance of the evidence indicates that the first observation of pteroylglutamic acid deficiency was made by Wills in human subjects and that her experiments were the first to differentiate pteroylglutamic acid from the erythrocyte maturation factor of concentrated liver extracts.

Other studies of blood dyscrasias of nutritional origin in monkeys; "vitamin M". From time to time, reports of experiments with monkeys on restricted
diets have revealed the occurrence of a nutritional deficiency syndrome which was characterized by oral, gastrointestinal and hemopoietic disturbances. It was pointed out in a recent review (25) that observations were made in 1919 (26) which described diarrhea and dysentery in monkeys on diets consisting largely of autoclaved rice. Attention was also drawn (25) to the reported appearance of the syndrome in several laboratories and under various dietary regimes.

A separate series of investigations with monkeys was started in 1935 (27). In an attempt to produce riboflavin-deficiency cataract, a cooked diet of polished rice, ground wheat and purified casein was used, supplemented with cod liver oil, salt mixture and orange. A deficiency syndrome was encountered which was marked by anemia and leucopenia, ulceration of the gums, diarrhea, and susceptibility to bacillary dysentery. The disease terminated fatally within 26 to 93 days if allowed to progress. It was prevented by brewers’ dried yeast, 10 grams daily or by a liver-stomach preparation, 2 grams daily (28).

It was reported (29) that riboflavin did not appreciably alter the course of the disease, termed “nutritional cytopenia”, and that nicotinic acid, 10 mgm. or 50 mgm. daily, did not prevent the development of the syndrome and did not postpone its fatal termination. The term “vitamin M” was proposed for the factor which prevented nutritional cytopenia in the monkey. Other features of the disease included edema (30) and susceptibility to various experimental infections (31, 32). The resistance of normal monkeys and the susceptibility of “vitamin-M-deficient” monkeys to bacterial dysentery was strikingly demonstrated (33).

An article from another laboratory (34) established the important point that leucopenia developed in monkeys which received a purified diet which was supplemented with certain vitamins, the effect of some of which had not been previously reported. The list included thiamine, riboflavin, pyridoxine, niacinamide, calcium pantothenate and ascorbic acid. The additional administration of 50 mgm. each of choline and para-aminobenzoic acid was ineffective. This report differentiated the protective factor from the known members of the vitamin B complex, with the possible exception of biotin. In addition, a new light was cast upon the possible nature of the unidentified factor; it was reported that a “folic acid concentrate” produced a white-cell response. As a result of assays of various supplements with Streptococcus fecalis R, doubt was later cast upon the possible identity of vitamin M with “folic acid” (25); this was, however, before studies with enzyme preparations had revealed the presence of microbiologically-inert conjugates of pteroylglutamic acid in natural foods. With the use of such enzyme preparations, prepared from rat liver, it was found that materials which were good sources of “vitamin M” were also good sources of “bound folic acid” which was liberated in the free form upon treatment with the liver preparation (35, 36) and the concept of the possible identity of the “S. lactis R stimulating substances together with the substances enzymatically convertible to such factors” with “vitamin M” was advanced (37). Finally it was demonstrated that pteroyltriglutamic acid, 4 or 4.5 mgm. per monkey in divided doses over a period of a few days, produced prompt and complete remis-
sion of nutritional cytopenia in monkeys, with a return of the granulocyte and total counts to normal levels, and with marked reticulocyte crises (19). Attention was drawn to the observation that the reticulocyte responses of the monkeys to pteroyltriglutamic acid were greater than the corresponding responses of pernicious anemia patients to adequate therapy (38). Monkeys on purified diets supplemented with all the known vitamin B factors except pteroylglutamic acid developed clinical and hematological signs of nutritional deficiency (20). Two of the animals were treated with pteroylglutamic acid, 0.1 to 0.35 mgm., at various intervals. Clinical and hematological remissions were observed.

Monkeys were fed a purified diet and a gradual loss of weight was observed followed by a syndrome which included anorexia, leucopenia and lowered resistance to secondary infections and which had a fatal termination. Feeding a norite eluate fraction of liver, which contained “folic acid”, was found to promote growth and to alleviate leucopenia (39). More recently, studies from the same laboratory have indicated that even when a “folic acid” concentrate was supplied, further addition of whole liver was necessary for optimum growth and “blood regeneration” in monkeys (40). The activity of the liver preparation was very easily destroyed by heating (41).

Monkeys were maintained on a purified diet deficient in pteroylglutamic acid and the effect of the deficiency on experimental poliomyelitis was studied (42). An increased resistance to the disease was observed when a subacute nutritional deficiency was produced by maintaining the animals on a suboptimal level of a liver “folic acid” concentrate, but no resistance was observed in “acute” pteroylglutamic acid deficiency.

It was stated (43) that a concentrate “showing high B10 and B11 activity” for the chick was inadequate as a source of folic acid for the monkey. Presumably “B10 and B11 activity” is an expression of the presence of conjugated pteroylglutamic acid, in which case this finding may be contrasted with the parallel drawn (35, 36) between vitamin M activity and the presence of “bound folic acid” which was liberated into a microbiologically active form by enzymic digestion.

Biological effects of xanthopterin. Rats three to four weeks old when fed exclusively on goats’ milk were found to develop an anemia which did not respond to supplementation with iron. The erythrocyte count fell to around 1 million per cu. mm. and the blood picture was of the macrocytic type (44). Typical reticulocyte responses were obtained with liver preparations and the condition was used as a method of testing such preparations (45). The animals showed sprue-like symptoms, so that the condition was termed “rat sprue”. In another investigation of goats’ milk anemia (46) fractionation of liver and human urine indicated that the active material had properties resembling those of uropterin (xanthopterin). Accordingly, a sample of uropterin was obtained from Kocchara. This was injected at various levels, the effect on the erythrocyte counts was followed and graded responses were obtained over a range of 0.5 to 1.0 microgram; 10 micrograms did not appear to cause a greater response than did 1 microgram and levels of 1 or 10 micrograms produced an elevation of 2 to 3
million in the erythrocyte count in 14 days. This experiment was the first clue to the chemical nature of pteroylglutamic acid. In contrast to the preceding report (44), the anemia was stated to be also relieved by iron and copper. Failure on the part of Rominger to confirm the results with uropterin was noted (47). However, a subsequent report (48) indicated that xanthopterin, 20 micrograms daily, produced a growth response and an increase in the white cell count in rats receiving a purified diet containing succinylsulfathiazole. Once again, difficulty in repeating these results was encountered (35, 49, 50, 51).

Xanthopterin was found to have a hemopoietic effect when 50 micrograms was injected into fingerling salmon with nutritional anemia (52). The salmon were made anemic by feeding "a high protein diet which contains yeast as a source of the vitamin B complex". The red cell counts per cu. mm. ranged from 416,000 to 916,000 for the control fish and from 659,000 to 1,305,000 for the injected fish 2 to 3 days after the injection.

A consistent but transitory response was obtained when monkeys with nutritional cytopenia were treated with xanthopterin 2.5 to 10 mgm. daily (53). The response consisted of a reticulocyte rise and a marked increase in both white and red cell counts, but the counts soon declined again, and three of the four animals died.

When fresh rat liver tissue was incubated with xanthopterin, a marked increase in the "folic acid" content of the preparation was obtained as compared with rat liver which was incubated alone or with leucopterin, adenine, guanine, xanthine, uracil or cytosine. The "folic acid" content was measured by assay with L. casei (50). This observation was confirmed (35), and it was noted that the liver tissue of monkeys, but not that of chicks, appeared to show a higher "folic acid" content when incubated with xanthopterin than when incubated alone. In another report, it was stated that the "folic acid" content of rat liver and muscle was influenced during digestion by various factors including neutral salts, cyanide, xanthopterin, the degree of dispersion of the tissue, the pH, the length of the digestion period and the addition of taka-diastase (54). A protective effect of xanthopterin against the destruction of "folic acid" by an enzyme in rat liver was suggested (55). Another obvious variable is the possible effect of the various factors upon the action of "vitamin B₆ conjugase" enzyme system (p. 79) present in rat liver, particularly evident in one experiment (54, fig. 4) in which rat liver, incubated alone at various pH values was found to yield a maximum amount of "folic acid" at pH 7.

**Pteroylglutamic acid in poultry nutrition.** A dietary deficiency of pteroylglutamic acid may readily be produced in young chicks on purified diets. Chicks appear to derive very little of their vitamin B-complex requirement from "intestinal synthesis", and they are, in contrast to rats, quite susceptible to a dietary lack of pteroylglutamic acid. The ease with which deficiencies were produced in chicks on purified diets led to the extensive use of this species as an experimental animal.

Starting in 1938 investigations were made to determine whether the chick needed B-complex vitamins in addition to the factors then known. The require-
ment of the chick for pyridoxine, nicotinic acid, choline and biotin was unexplored at that time. In one report (56), rice bran extract and a fullers' earth adsorbate of whey were added to a simplified basal diet. Growth was improved by a water-soluble factor which was present in alfalfa meal and which was precipitated by alcohol and adsorbed to some extent by fullers' earth, from which it was eluted by a mixture of water, acetone, and ammonia. It was found (6) that chicks grew slowly on a diet consisting principally of polished rice and washed fish meal, supplemented with thiamine and whey adsorbate, and with filtrate factor (pantothenic acid) preparations from rice bran or whey. Growth was greatly increased by yeast, or by alfalfa meal or a water extract of it. The factor, termed "Factor U", extracted from yeast, was adsorbed on fullers' earth and eluted by a mixture of pyridine, alcohol and water. Evidence for the presence of an unidentified factor in cereals, yeast and milk was advanced (57). The factor was needed for growth and hatchability, and was destroyed by prolonged dry heat. Biotin is destroyed by such treatment (58). In a further report on "Factor U" (59), pyridoxine was found to promote growth when added to the basal diet, but additional growth was produced by adding the yeast fullers' earth eluate. The presence of a growth-promoting factor in yeast distinct from thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and choline was noted (60).

In another investigation (7), acid-hydrolyzed yeast, rice polishings extract, and alcohol extract of liver were used as supplements to a purified diet. Presumably the pteroylglutamic acid in the yeast was destroyed by the acid hydrolysis. A macrocytic hypochromic anemia was developed by the chicks on this diet; it was stated that evidence indicated that it was not prevented or cured by any vitamin previously described. The antianemic factor from an undescribed source was adsorbed on fullers' earth at pH 1. The factor was given the name "vitamin B_6" (61). This investigation was important in that it reported the specific anti-anemic properties of the new vitamin and thus foreshadowed future developments.

Strong evidence that the norite eluate factor required by L. casei was essential for the chick was reported (62). A concentrate of the factor had a marked growth-promoting effect when added at a level of only 0.1 gram per kilo of diet. The "norite eluate factor" for L. casei is now presumed to be pteroylglutamic acid. It was found that disparities existed between the microbiological potency of certain concentrates and their activity for chicks. Apparently on this basis, the existence of two new vitamins, B_10 and B_11, active for chicks, was postulated (11). "Vitamins B_10 and B_11" were the subject of a number of publications (63, 64, 65) but in a subsequent report from the same laboratory (66) it was noted that "neither para-aminobenzoic acid nor vitamins B_10 + B_11 gave a supplementary effect in the presence of synthetic folic acid". Presumably "vitamins B_10 and B_11" represented a conjugate or conjugates of pteroylglutamic acid.

The biological activity of crystalline pteroylglutamic acid for the chick was first reported in 1943 (67). "Vitamin B_6" from liver was fed at a level of 2.5 parts per million of diet which prevented anemia and enabled good growth to
take place. The chicks which received the purified diet plus pteroylglutamic acid grew more rapidly than chicks on a "broiler ration". It was indicated (68) that 0.4 parts per million of diet was sufficient for normal hemoglobin, hematocrit, red cell count and thrombocyte values, but about a level of 4 parts was required for the production of normal leucocyte levels. A comparison of injected and orally administered dosage (69) indicated that subcutaneous injection was, if anything, slightly more effective than feeding by pipet. In further studies, it was reported that maximum growth, with submaximal hemoglobin response, was obtained by adding pteroylglutamic acid, either free or as the conjugate, at a level of 0.25 parts per million of diet (70).

The pteroylglutamic acid requirement of chicks was studied on a purified diet using growth, feathering and pigmentation as criteria (71). The vitamin was administered by injection 5 times weekly and it was found that a level calculated to supply 10 micrograms per day produced feathering and pigmentation equivalent to that observed in chicks receiving 10 per cent of brewers' yeast as a supplement to the basal diet but growth was somewhat more rapid on the yeast-supplemented diet. Growth and feathering were in proportion to the level of pteroylglutamic acid used.

Chicks on a purified diet were found to need at least one part of pteroylglutamic acid per million parts of diet for normal feather pigmentation and two parts per million to give growth equal to that obtained with a liver fraction (72). Pteroylglutamic acid, added at levels of one to five parts per million of purified diet, was found to be essential for the normal growth of feathers in chicks (73). The effect was not lessened by adding sulfamerazine or certain other "intestinal antiseptics".

In experiments with chicks on a purified diet, pteroylglutamic acid was found to produce maximum growth and hemoglobin values when added at a level of 0.5 to 1.0 mgm. per kilo of diet (74). Chicks were found to need 0.25 part of pteroylglutamic acid per million parts of purified diet for growth, feathering and hemoglobin formation (66). When sulfasuxidine was added to the diet the requirement was increased to between 0.5 and 1.0 part per million. In contrast, the pteroylglutamic acid requirement of chicks was found not to be increased by the addition of sulfasuxidine to a purified diet (75). Chicks were found to require 0.45 part of pteroylglutamic acid per million parts of diet for growth and hemoglobin formation to four weeks of age. The requirement for hemoglobin formation to six weeks of age was stated to be slightly less, 0.35 part per million and 0.55 part per million were required to produce normal feathering at six weeks of age. Attention was drawn (76) to earlier low estimates of the pteroylglutamic acid requirement of chicks (11, 63, 65, 77) which had resulted from feeding preparations of pteroylglutamic acid conjugates. It was pointed out that microbiological assays of such conjugates, even after treatment with taka-diastase, did not reveal the total pteroylglutamic acid content.

Perosis was observed in chicks on a purified diet without pteroylglutamic acid and the incidence was increased by adding 2 per cent of sulfasuxidine. The perosis was prevented by adding 0.2 part per million of pteroylglutamic acid
to the diet without sulfasuxidine and 0.3 part per million to the diet containing sulfasuxidine. The higher incidence of perosis on the sulfonamide-containing diet led to the suggestion that pteroylglutamic acid stimulated the intestinal flora to produce an unknown anti-perotic factor (78).

A relation was observed between pteroylglutamic acid deficiency in chicks and an absence of the normal response to the administration of diethylstilbestrol (79). Large doses of stilbestrol produced an only slight increase in the weights of oviducts of young chicks maintained on a purified diet which was deficient in pteroylglutamic acid, but stilbestrol produced a marked increase in the weight of the oviducts of control chicks receiving a supplement of 20 micrograms of pteroyltiglutamic acid daily. The failure of the deficient birds to respond was not due merely to inanition, for chicks with pantothenic acid deficiency and of comparable body weights showed substantial responses to stilbestrol as measured by increase in the weight of the oviduct.

Differentiation of pteroylglutamic acid from the erythrocyte maturation factor of concentrated liver extracts, previously indicated by studies with human subjects and with monkeys (14, 15, 16), was further emphasized by observations with chicks. In one investigation, an undescribed preparation of the anti-pernicious-anemia factor was found to be ineffective in the prevention of "vitamin B\textsubscript{12} deficiency" (80) and subsequently (81) it was reported that pteroylglutamic acid was not liberated in appreciable quantities from concentrated liver extract by treatment with "conjugase" as supplied by dried chicken pancreas. The maturation factor as present in liver extract was ineffective for growth when injected into pteroylglutamic acid-deficient chicks at a level which would correspond, on an anti-pernicious-anemia basis, to about 0.4 mgm. of pteroylglutamic acid daily.

Reproduction in chickens, previously not reported with purified diets, was obtained on a diet which contained pure vitamin B complex factors including pteroylglutamic acid (82).

Young turkeys were found to develop marked nutritional deficiency on diets deficient in pteroylglutamic acid. A spastic type of cervical paralysis was observed on purified diets which were deficient in the vitamin (83). The paralysis was reversed by the administration of pteroylglutamic acid. These observations were confirmed (84) and the occurrence of a blood dyscrasia characterized by macrocytosis and elongation of the erythrocytes was described. The requirement for pteroylglutamic acid under the conditions of the experiment appeared to be at least twice as great for turkeys as for chickens.

Studies of the effects of pteroyltiglutamic acid in chicks. This compound was found to be "active in the nutrition of the chick" (85).

It was reported that pteroyltiglutamic acid, when added at the rate of 0.5 mgm. per kilo of purified diet, was only partially effective in promoting growth and preventing anemia (86). However, when the lactone of either 5-pyridoxic acid (2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxy pyridine) or 4-pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethyl pyridine) was added at a level of 0.5 mgm. per kilo to the diet together with pteroyltiglutamic acid,
termed *L. casei* factor, at the same level, a marked gain in weight was produced and anemia was completely prevented. The names alpha and beta "pyracin" were proposed for the two pyridoxic acids.

Hens on a commercial diet were made anemic by bleeding, and the rate of regeneration of hemoglobin was followed (87). The injection of either 4-pyridoxic acid or pteroyltriglutamic acid, 50 micrograms daily, hastened the rate of the hemoglobin level, and when these supplements were administered together, the rate was still further increased.

In another communication, the incubation of pteroyltriglutamic acid with fresh chick liver was found to result in an increase in "folic acid content" as measured with *S. fecalis* R. There was a further increase when 4- or 5-pyridoxic acid was added but a diminution when pyridoxic acid lactone was added rather than the acid (88). Pteroylglutamic acid was found to be effective against anemia in chicks without the addition of pyridoxic acid lactone to the diet (89, 76). Evidence was obtained with chicks (90) which tended to confirm the report of the supplementary effect of 4-pyridoxic acid on the utilization of pteroyltriglutamic acid. The experimental data were meager due to the small amount of pteroyltriglutamic acid which was available.

Other workers reported that pteroyltriglutamic acid without added pyridoxic acid was an effective as pteroylglutamic acid, on a molar basis, in promoting growth and preventing anemia in chicks (74) (91). As compared with pteroylglutamic acid, pteroyltriglutamic acid appeared to be completely utilized with or without the addition of 4-pyridoxic acid by chicks on a diet similar to that described by the Cornell group (91). It has also been found that pteroylheptaglutamic acid was utilized as efficiently as pteroylglutamic acid, without the addition of pyridoxic acid, by chicks on a purified diet (92).

Role of pteroylglutamic acid in microbiological nutrition. A summary of the pteroylglutamic acid requirements of microorganisms is given in table 3.

A large number of lactic acid bacteria have been shown to need pteroylglutamic acid for growth. In an investigation of the nutritive requirements of lactic acid bacteria (8) it was shown that an unidentified growth factor existed which later proved to be pteroylglutamic acid. Later it was shown at the same laboratory that this factor was required by other lactic- and propionic-acid-forming bacteria *Streptococcus fecalis* R, *Lactobacillus delbruckii*, and *Propionibacterium pentosaceum* (93).

*Clostridium tetani* was found to require a growth factor which could be replaced by concentrates of pteroylglutamic acid made from liver (94) or by a "folic acid" preparation obtained from spinach (95). Three "folic acid" preparations of widely varying potencies as measured with *S. fecalis* R were found to have corresponding activities for *Cl. tetani* thus indicating that pteroylglutamic acid was the effective agent.

The growth requirements of a large number of enterococci was investigated (96) and it was found that of 43 organisms investigated 9 required pteroylglutamic acid. It was also found by the same investigators that of 21 strains of *Streptococcus lactis* none required pteroylglutamic acid (97). The requirements
of a large number of lactic acid bacteria for pteroylglutamic acid and the "S. L. R factor" were investigated (99, 104) and are described in detail elsewhere in this review.

The nutrition of *Tetrahymena geleii* has been thoroughly investigated and this organism was shown to require pteroylglutamic acid (106, 110). The amount required for half maximum growth is 0.00065 microgram per ml. of culture medium which is about three times the amount required for half maximum growth by *S. fecalis* R. (10). The "S. L. R factor" which is active for *S. fecalis* R but relatively inactive for *L. casei* was found to be about 0.2 per cent as active as

### TABLE 3

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<tr>
<th>MICROORGANISMS</th>
<th>PTEROYLGLUTAMIC ACID</th>
<th>PTEROYL-TRI-GLUTAMIC ACID</th>
<th>PTEROYL-HEPTAGLUTAMIC ACID</th>
<th>PTERIC ACID</th>
<th>S. L. R. FACTOR</th>
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<tr>
<td><em>Clostridium tetani</em></td>
<td>+ (94, 95, 100)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>+ (67)</td>
<td>+ (85)</td>
<td>- (70)</td>
<td>- (101)</td>
<td>- (102)</td>
<td>+ (103)</td>
</tr>
<tr>
<td><em>L. helveticus</em></td>
<td>+ (93)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. delbruckii</em></td>
<td>+ (93)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. delbruckii LD5</em></td>
<td>+ (104)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>L. bulgaricus D5</em></td>
<td>+ (104)</td>
<td></td>
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</tr>
<tr>
<td><em>Propionibacterium pentosaceum</em></td>
<td>+ (93)</td>
<td></td>
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</tr>
<tr>
<td><em>Streptococcus durans</em> 98A</td>
<td>+ (104)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>S. fecalis</em> 732</td>
<td>+ (104)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>S. fecalis</em> F-24</td>
<td>+ (104)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>S. fecalis</em> R</td>
<td>+ (104)</td>
<td>± (85)</td>
<td>- (70)</td>
<td>+ (101)</td>
<td>+ (104)</td>
<td>+ (105)</td>
</tr>
<tr>
<td><em>S. fecalis</em> S-108A</td>
<td>+ (104)</td>
<td></td>
<td></td>
<td>- (104)</td>
<td>+ (105)</td>
<td></td>
</tr>
<tr>
<td><em>S. zymogenes</em> 5Cl</td>
<td>+ (104)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tetrahymena geleii</em></td>
<td>+ (106)</td>
<td></td>
<td></td>
<td>+ (107)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast &quot;Old process&quot;</td>
<td>+ (109)</td>
<td></td>
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</tr>
</tbody>
</table>

* Approximately 0.2 per cent as active as pteroylglutamic acid.

pteroylglutamic acid for *T. geleii* (110). Later it was reported by the same workers (108) that pteroylheptaglutamic acid was active. This contrasts *T. geleii* with *S. fecalis* and *L. casei* which are unable to utilize this conjugate. Thus the ability of *T. geleii* to utilize the conjugate is similar to that of animals, and parallels the similarity of *T. geleii* and the higher animals in amino acid requirements (111).

It seems likely that those organisms which do not require pteroylglutamic acid are able to synthesize it. *Bacillus lactis* *acidi*, *Lactobacillus arabinosus*, *Lactobacillus pentosus*, *Bacillus brassicace*, *Leucosnosto* *mevnteroides* and *Lactobacillus gayonii* are not stimulated by pteroylglutamic acid but were able to synthesize significant amounts of the factor (93).
The synthesis of pteroylglutamic acid by five microorganisms was reported (112). The assays were made after papain and takadiastase digestion of the cells and using S. fecalis R as the assay organism. This enzyme treatment would presumably not hydrolyze the conjugates present so the results must be interpreted to measure primarily free pteroylglutamic acid and any S. L. R factor which may be present. The organisms were grown on a purified media containing no pteroylglutamic acid and the cells and media assayed separately. The results were as follows:

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>MICROGRAMS PTEROYLGLUTAMIC ACID FOUND PER GRAM DRY CELLS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobacter aerogenes (aerobic)</td>
<td>5 31</td>
</tr>
<tr>
<td>Aerobacter aerogenes (anaerobic)</td>
<td>2 7</td>
</tr>
<tr>
<td>Serratia marcesans</td>
<td>6 28</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>3 23</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>7 7</td>
</tr>
<tr>
<td>Clostridium butylicum</td>
<td>1 6</td>
</tr>
</tbody>
</table>

* Calculated on the basis of pteroylglutamic acid having a "potency" of 137,000.

The growth-promoting activity of pteroylglutamic acid for S. fecalis R was reported to be reversed by the addition of a synthetic product, "methylfolic acid", which was prepared by reacting 2,4,5-triamino-6-hydroxy pyrimidine and p-aminobenzoyl d(-)-glutamic acid with 2,3-dibromobutyraldehyde (113).

A relationship was established between pteroylglutamic acid synthesis and sulfanilamide in E. coli (114). A sulfonamide resistant and a sensitive strain of E. coli were grown in concentrations of sulfanilamide which permitted some growth. Both strains produced less pteroylglutamic acid when grown in the presence of sulfanilamide than in the absence of it. Biotin synthesis on the other hand was not greatly affected by the presence of the drug which demonstrated that the sulfanilamide did not exert a general depressing action on synthesis of all the vitamins.

While pteroyltriglutamic acid and pteroylglutamic acid were approximately equally active for L. casei, the shapes of their respective response curves were different (115). There was a lag in the response to low concentrations of pteroyltriglutamic acid which resulted in a sigmoid shaped curve. The lower part of the response curve with pteroylglutamic acid approached a straight line. A sample of "folic acid, potency 3100" obtained from spinach gave the same shape response curve as that of pteroylglutamic acid, and on this basis it was suggested that the active constituent in the "folic acid" preparation was pteroylglutamic acid. Comparative assays with L. casei and S. fecalis R showed the "folic acid" preparation to have the same relative potency as pteroylglutamic acid for these two organisms. By using a folic acid preparation with potency standardized in terms of a liver fraction the "potency" of pteroylglutamic acid was estimated to be approximately 137,000.
Para-aminobenzoic acid and its relation to pteroylglutamic acid in bacterial nutrition. There is evidence that p-aminobenzoic acid functions as a precursor of pteroylglutamic acid in the nutrition of certain bacteria. The bacteriostatic effect of the sulfonamides is due in the case of some microorganisms to inhibition of the enzyme system which synthesizes pteroylglutamic acid from p-aminobenzoic acid.

A possible indication of the relation between p-aminobenzoic acid and pteroylglutamic acid appeared before the chemical nature of pteroylglutamic acid was revealed. It was reported (116) that a yellow pigment was formed in cultures of a certain strain of Mycobacterium tuberculosis when it was grown in media containing high concentrations of p-aminobenzoic acid, and it was suggested that the pigment might be related to the vitamin B complex. It was also suggested that the enzyme responsible might be a specific oxidase (117). The pigment was reported to contain oxidized aromatic nitrogen groups as indicated by an increase of aromatic amino nitrogen after treatment with zinc. The pigment gave negative results for the presence of “folic acid” when tested with S. fecalis R. This negative result presumably would not exclude the possible presence of certain conjugates of pteroylglutamic acid which are known to be of quite low activity for S. fecalis R. Pteroylheptaglutamic acid (Table 1) is an example of such a conjugate. The formation of the pigment could be lessened or inhibited by sulfanilamide (118). It was noted that a similar culture of this strain of M. tuberculosis contained a fraction which promoted growth in chicks. This effect was attributed to considerable amounts of “B10 and B11” rather than to pteroylglutamic acid (64); the relation between pteroylglutamic acid and “B10 and B11” (66) has not then been evaluated. It thus appeared possible that an effect of p-aminobenzoic acid upon this strain of M. tuberculosis was to increase the production of conjugated pteroylglutamic acid. The nature of the yellow pigment (116) has not yet been described.

A mixed culture of organisms was obtained from the duodenum of chicks. The growth rate of the culture was increased by p-aminobenzoic acid to the medium, and simultaneously a three-fold increase in folic acid production as measured by assay with S. fecalis R was observed (119).

It was noted that the growth of S. fecalis R and L. casei in the presence of pteroylglutamic acid, pteroyltriglutamic acid or pteroic acid was affected very little by sulfonamides (120). Growth of these organisms did not take place if p-aminobenzoic acid or p-aminobenzoyl glutamate was substituted for pteroylglutamic acid, pteroyltriglutamic acid or pteroic acid. This indicated that these organisms cannot form pteroic acid or pteroylglutamic acid from p-aminobenzoic acid or p-aminobenzoyl glutamate and that they need preformed pteroic acid or pteroylglutamic acid. Hence there is no opportunity for a sulfonamide to compete with p-aminobenzoic acid in the nutrition of these organisms. With another organism, S. fecalis Ralston, which responded to p-aminobenzoic acid, p-aminobenzoyl glutamate, pteroylglutamic acid, pteroyltriglutamic acid or thymine, competitive inhibition between sulfadiazine and p-aminobenzoic acid or p-aminobenzoyl glutamate was observed. In the presence of pteroylglutamic acid,
pteroyltriglutamic acid or thymine, the organism became highly resistant to sulfadiazine, indicating that the inhibitory action of sulfadiazine was due to interference with the formation of pteroylglutamic acid from p-aminobenzoic acid and that when preformed pteroylglutamic acid was supplied, the organism did not "need" to carry out this synthesis and hence sulfadiazine was noninhibitory. The observations with thymine recall the previous observation that for certain bacteria this substance in the absence of pteroylglutamic acid is a growth promoting factor, and the suggestion that the function of pteroylglutamic for these bacteria is to catalyze the formation of thymine (p. 66). A third type of organism, represented by E. coli, did not require preformed pteroylglutamic acid, and the bacteriostatic effect of sulfadiazine on this type was reversed by p-aminobenzoic acid but not by pteroylglutamic acid, which observation might be presumed to indicate that these organisms could not utilize preformed pteroylglutamic acid.

Thymine and pteroylglutamic acid. A relation between the pyrimidine base, thymine (5-methyl uracil) and "folic acid" was observed in studies with S. fecalis R (121). This lactic acid organism was enabled to grow by the addition of both thymine and a purine base. Thymine could not be replaced by uracil, but the purine requirements were less specific, adenine and guanine being almost interchangeable.

Thymine was also found to have a growth-promoting effect on L. casei (103). A combination of both thymine and a purine base was necessary. Approximately 0.5 microgram of thymine and 5.0 micrograms of guanine per ml. of media were required to give the maximum response. The maximum growth obtained with thymine and purines was only half that obtained with concentrates of L. casei factor (pteroylglutamic acid). Thymine could not be replaced by uracil or cytosine while the purine requirements were met by guanine, xanthine, adenine or hypoxanthine.

A report on the requirements of S. fecalis R showed that thymine could not be replaced by 4-carboxyuracil, 4-carboxythymine, or 5-carboxyuracil (109). Nucleic acid was found to be inactive as a source of thymine for S. fecalis (122), indicating the inability of the organism to utilize thymine in the form of a nucleic acid. While no data has appeared on the activity of thymine nucleotide, the nucleoside thymidine has been shown to be as active as thymine on a molar basis (105). In this report the inactivity of a number of pyrimidines was also described.

The interfering effect of thymine on the assay of pteroylglutamic acid with L. casei received comment (123).

A possible role of thymine in serving as a substitute for "folic acid" was formulated on the basis of experiments with these two compounds in the nutrition of S. fecalis R and L. casei (105). It was found that while pteroylglutamic acid alone would give a response, addition of a purine was needed to give maximum response. In the presence of a purine, approximately 5,000 times as much thymine as pteroylglutamic acid was required. With S. fecalis R, thymine permitted the same maximum growth as was obtained with pteroylglutamic acid,
but with *L. casei* thymine plus a purine gave only half the maximum response produced by pteroylglutamic acid.

The possibility of formation of pteroylglutamic acid by *S. fecalis* R cells during growth on thymine was tested by autoclaving the cells with dilute hydrochloric acid and assaying with *L. casei*. A "plateauing" of the *L. casei* assay response at approximately half-maximum growth was characteristic of the response obtained with thymine and suggested that the material in the *S. fecalis* R cells giving the response was thymine and not pteroylglutamic acid. These observations led to the suggestion that pteroylglutamic acid functioned directly or indirectly as a coenzyme in the synthesis of thymine or related compounds by *S. fecalis* R. Three to four micrograms of thymine were taken up per mg. of *S. fecalis* R cells during growth, an amount which may be compared with the value of about 2.0 micrograms of thymine present per milligram of dried tubercle bacillus (124).

Studies of the effect of a large number of pyrimidines on the growth of *L. casei* were reported (125). Derivatives of thymine in which one or both of the oxygen atoms were replaced by an imino or thio group in many cases promoted the growth of *L. casei*. The replacement of oxygen by the imino group appeared to lower the activity to about 0.1 that of the corresponding oxy compound. Other relationships of the effect of chemical structure upon biological activity were discussed. It was found that isobarbituric acid and 5-amino uracil reversibly antagonized the growth promoting effect of thymine or pteroylglutamic acid. It was also observed that bromouracil could inhibit completely the growth of *L. casei* with thymine as the nutrient but had no effect or produced slight stimulation when pteroylglutamic acid was used as a nutrient. This observation did not support the hypothesis (105) that the function of pteroylglutamic acid in promoting the growth of lactic acid bacteria was to enable the organisms to synthesize thymine. It was further observed (125) that nitouracil at certain concentrations could antagonize the growth-promoting action of pteroylglutamic acid but had no effect on the growth-promoting action of thymine. Thymine in large doses has been reported to cause hemopoietic remission in pernicious anemia and sprue (pp. 84, 90); the substance appears to be ineffective in preventing pteroylglutamic acid deficiency in rats and chicks (126, 127).

The "*Streptococcus lactis* R factor". A factor was described which was highly active for *S. fecalis* R, but relatively inactive for *L. casei* (102). One microgram of this "*S. L. R factor" had an activity for *S. fecalis* R equal to 1.25 micrograms of "folic acid (potency 137,000)" but for *L. casei* it had an activity equal to less than 0.00001 microgram of the same preparation of folic acid. Pteroylglutamic acid has been shown to have the same potency as a "folic acid preparation of potency 137,000" (115).

A survey was made of the "*S. L. R factor" and pteroylglutamic acid requirements of a number of lactic acid bacteria (104). The results which appear in table 4 revealed that those organisms which are able to utilize the "*S. L. R factor" can also use pteroylglutamic acid. A certain number, mostly Lactobacilli, were able to utilize pteroylglutamic acid only.
These results also demonstrated that different strains of the same organism have widely differing requirements. Thus one strain of *S. fecalis* can use either the “S. L. R factor” or pteroylglutamic acid, another requires pteroylglutamic acid while a third requires neither. Synthesis of pteroylglutamic acid was stated to be established for three of these strains.

It was also reported that certain enterococci were able to convert the “S. L. R factor” into a form which is active for *L. casei*. It was found that 0.06 microgram of this factor per 10 ml. of media did not permit growth of *L. casei* but that 1 ml. of a fluid culture of “*S. fecalis*” grown with 0.006 μg per ml. of the factor would permit rapid growth. This demonstrated that *S. fecalis R* had converted the “S. L. R factor” into a form active for *L. casei*, which was presumed (104) to be pteroylglutamic acid. In the same report it was stated that incubation of the “S. L. R factor” with rat liver suspensions did not give rise to “folic acid”.

### TABLE 4

**Streptococcus lactis R factor and pteroylglutamic acid requirement of lactic acid bacteria**

<table>
<thead>
<tr>
<th>Organisms requiring:</th>
<th>PTEROYLGLUTAMIC ACID</th>
<th>NEITHER</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus lactis R</em></td>
<td><em>Lactobacillus casei</em></td>
<td><em>Lactobacillus arabinosus</em> 17-5</td>
</tr>
<tr>
<td><em>Streptococcus fecalis 732</em></td>
<td><em>Lactobacillus delbrueckii</em> LD5</td>
<td><em>Leuconostoc mesenteroides</em> 6205</td>
</tr>
<tr>
<td><em>Streptococcus fecalis F24</em></td>
<td><em>Lactobacillus bulgaricus</em> O5</td>
<td><em>Streptococcus lactis</em> 374, 4487, 8039, 7963, 4386, L103, L104, L206</td>
</tr>
<tr>
<td><em>Streptococcus zymogenes 5Cl</em></td>
<td><em>Streptococcus casei</em> 19</td>
<td><em>Streptococcus fecalis</em> 10Cl</td>
</tr>
<tr>
<td><em>Streptococcus durans 98A</em></td>
<td><em>Streptococcus fecalis</em> S108A</td>
<td><em>Streptococcus zymogenes</em> 6054</td>
</tr>
</tbody>
</table>

This constituted evidence for the belief that the “S. L. R factor” is not a conjugate of pteroylglutamic acid since liver enzymes are capable of cleaving conjugates.

In a later paper from the same laboratory a detailed study of the conversion of the “S. L. R factor” to pteroylglutamic acid by various enterococci was reported (99). The ability of these organisms to effect this conversion varied widely. Resting cell suspensions of these organisms in phosphate buffer were used. A 10 ml. suspension of *S. lactis R* or *S. zymogenes* converted 5 micrograms of S. L. R factor to about 1.0 microgram of pteroylglutamic acid1 in three hours while *S. fecalis* 232 and *S. durans* 98A formed only 0.18 microgram. The addition of carbohydrates, e.g. d-ribose, glucose and fructose increased the formation of pteroylglutamic acid 5- to 15-fold. Other carbohydrates tested permitted growth but did not accelerate the formation of pteroylglutamic acid. Those

1 The data have been recalculated on the basis of pteroylglutamic acid assuming that pteroylglutamic acid has the same activity as folic acid of potency 137,000.
sugars which could act as hydrogen acceptors as evidenced by reduction of methylene blue during the incubation with resting cells, were the ones capable of increasing the rate of conversion. Sucrose was unable to stimulate this conversion by resting cells which had been grown on glucose. However, if the organism were initially grown on sucrose, then sucrose was as efficient as glucose in stimulating the conversion of the S. L. R factor, and methylene blue was reduced by the product obtained when sucrose was incubated for three hours with resting cells.

In the assay of pteroylglutamic acid formed by conversion from the "S. L. R factor" the entire cell suspensions were added directly to the assay medium without any preliminary treatment. Practically all this activity was found to be within the cells. When these cells were autoclaved with water only 12 per cent of the activity was recovered and only 28 per cent when autoclaved with phosphate buffer. However, when the cells were autoclaved in the assay medium, in 0.5 per cent sodium thioglycollate solution or in 5 per cent ascorbic acid and then assayed, complete recovery was obtained. This suggests that reducing substances exerted a protective influence during extraction from the cell. However, the residual pteroylglutamic acid in the liquid phase after autoclaving was resistant to further autoclaving. These results emphasize the difficulties that can be encountered in assaying microbial cells and the care needed to assure complete extraction.

The "S. L. R factor" ("rhizopterin") was characterized as 4(((2-amino-4-hydroxy-6-pteridyl)methyl)formamido) benzoic acid; a compound of pteroic and formic acids (306). It is inactive for hemopoiesis in sulfonamide-treated rats and pteroylglutamic acid-deficient chicks (128). Pteroic acid is similarly inactive for the chick (1). There appears to be a small difference in the relative potencies of the S. L. R factor and pteroic acid for S. fecalis R and L. casei. The activities of the "S. L. R factor" when directly compared with pteroylglutamic acid have not been reported and the only evidence available is based on recalculation of the data obtained with "folic acid" with a potency of 40,000 obtained from spinach. Using this data, the "S. L. R factor" is 125 per cent as active as pteroylglutamic acid for S. fecalis R and only 0.001 per cent as active for L. casei. Pteroic acid is 50 to 100 per cent as active as pteroylglutamic acid for S. fecalis R depending on the time of incubation (127) and 0.01 per cent as active by L. casei assay.

*Pteroylglutamic acid in the nutrition of rats.* On purified diets which do not contain pteroylglutamic acid, the dietary need of the rat for this factor appears to be satisfied by the production of pteroylglutamic acid by the intestinal bacteria. The addition of any of several sulfonamides to the diet depresses the growth of the intestinal bacteria and results in the appearance of certain deficiency syndromes, one of which is characterized by agranulocytosis, leukopenia, anemia and slow growth. Administration of pteroylglutamic acid or of its conjugates cures this syndrome. Some difficulties of interpretation were encountered until the fact was appreciated that certain conjugates are microbiologically inactive. A relationship may exist between the utilization of pantothenic acid and pteroylglutamic acid in rats on such diets. Lactation leucopenia
due to pteroylglutamic acid deficiency has been observed in rats on purified
diets.

It was found that rats would grow and reproduce on a purified diet (129).
Shortly thereafter, it was shown that when sulfaguanidine was added to a similar
diet, the growth of young rats was greatly reduced and could be restored by the
addition of liver extract (130). A concentrate of “folic acid” was fed at the rate
of 5 milligrams daily to rats on a purified diet containing 1 per cent of sulfasuxi-
dine (131). Definite growth responses were observed when biotin was also fed.
Similar responses were obtained with a more concentrated “folic acid” prepara-
tion. In addition to promoting growth (132), “folic acid” concentrates were
found to restore the color of the hair in black rats which had become grey on diets
containing sulfaguanidine. The depressed growth under such conditions was
found to be counteracted by feeding yeast, yeast extract or rat feces (133).
Specific signs were described in rats on such diets (134), including agranulocytosis,
leucopenia, anemia and hypocellularity of the bone marrow. The changes were
prevented or reversed by a liver fraction which was precipitated from aqueous
solution by addition of 80 per cent ethanol. A crude norite eluate containing
“folic acid” was found to improve the leukocyte picture (49). These findings
were confirmed with a “folic acid concentrate” (135) which was found to exert
an effect similar to that of liver extract in promoting growth and preventing
leucopenia in rats on diets containing sulfasuxidine. In another investigation
(136) signs of pantothenic acid deficiency were observed in rats on purified diets
with sulfasuxidine and the pantothenic acid content of the liver tissue of the rats
was lowered, although the diet contained 40 parts of pantothenic acid per million.
When biotin and a “folic acid” concentrate were added to the diet, growth was
improved and the pantothenic acid content of the liver was increased. These
additions were also reported to lower the prothrombin time (137). The signs of
pantothenic acid deficiency, including porphyrin-caked whiskers, were found to be
removed by supplementation with biotin and a “folic acid” concentrate (138),
but not by additional amounts of pantothenic acid.

It was shown (50) that pteroylglutamic acid or pteroyltrimethylglutamic acid, 20
micrograms per day for 4 days, were effective in markedly increasing the granulo-
cytes and the total white cell count, and in relieving the anemia occurring in rats
on purified diets containing sulfaguanidine or sulfasuxidine.

The anemia induced by bleeding was studied in rats which were fed a purified
diet containing sulfasuxidine (139). The administration of pteroyltrimethylglutamic
acid was found to have a preventive and corrective effect on the anemia as
judged by measurement of hemoglobin concentration, hematocrit readings and
white counts. These observations may indicate a need for further studies of
the possible role of pteroylglutamic acid in the regeneration of hemoglobin fol-
lowing bleeding.

Attention was drawn to the unexpectedly high activity of milk in preventing
“folic acid” deficiency in rats on a purified diet containing sulfasuxidine (140).
The “folic acid” content of the milk was found to be quite low as determined by
assay with L. casei and S. fecalis R. When the milk was treated with “con-
jugase” the “folic acid” content was found to be increased more than 20-fold
which indicated that the preceding observation could be explained on the basis of utilization of conjugated pteroylglutamic acid by the rat.

Storage of pteroylglutamic acid in the liver of rats was reduced by adding sulfasuxidine to a purified diet and the folic acid content of the liver was increased when a liver fraction was added to the diet (142). A series of investigations (143, 144, 145, 146) emphasized the relationships between pteroylglutamic acid and other dietary ingredients. It was shown that granulocytopenia due to pteroylglutamic acid deficiency was obtained in a small percentage of rats which were fed purified diets without sulfonamides. The dyscrasia was also produced by feeding purified diets which were low in pantothenic acid; under these conditions anemia, leucopenia, granulocytopenia, and bone marrow hypoplasia were prevented by adding pantothenic acid. It was considered that the primary deficiency producing granulocytopenia under these experimental conditions was that of pteroylglutamic acid. The administration of pantothenic acid appeared to prevent the development of pteroylglutamic acid deficiency. Riboflavin-deficient rats were found to become anemic, granulocytopenic or both. The granulocytopenia was corrected by pteroylglutamic acid and the anemia, somewhat less consistently, by riboflavin. Severe granulocytopenia and anemia were produced in rats fed protein-free diets. The condition was prevented by casein but not by pteroylglutamic acid or pteroyltriglutamic acid. Casein did not correct the granulocytopenia but when casein and pteroylglutamic acid were both added the granulocytopenia was cured.

Anemia, leucopenia, and hemorrhage and necrosis of the adrenals were observed in rats on a purified diet containing thiourea. The leucopenia and granulocytopenia were not prevented by thyroid powder or thyroxin but were corrected by treatment with pteroylglutamic acid. Pteroyltriglutamic acid, 100 micrograms daily, was effective, but treatment for 4 days with 24 micrograms did not consistently correct the dyscrasia (147). These results may be contrasted with the reported ineffectiveness of pteroylglutamic acid in preventing the onset of agranulocytosis in two patients receiving thiouracil (148).

A description of the pathology of the bone marrow of rats as affected by pteroylglutamic acid deficiency was given (149). In the deficiency the marrow was hypocellular and showed general depletion especially of the myeloid series, although the erythroid series was usually also depleted even in the absence of anemia. Administration of pteroylglutamic acid resulted in a characteristic proliferation and regeneration and the marrow returned to normal after temporary over-compensation.

Hypochromic anemia was induced by promin and promizole in young rats on a purified diet. The administration of pteroylglutamic acid, 18 micrograms daily, exerted an anti-anemic effect (150).

It was reported that concentrates of pteroylglutamic acid improved the performance of rats during lactation (151). "Lactation leucopenia" in rats on purified diets was found to be partially prevented by addition of brewers’ yeast or liver extract (152) and subsequently pteroylglutamic acid was found to have
a similar effect (153) although the effect was not as complete as that of a liver fraction.

Intestinal synthesis of "folic acid" in rats on purified diets, was studied by examination of the cecal contents (154). Dextrin produced the largest amount of synthesis. A marked decrease in synthesis was caused by adding 2 per cent sulfathalidine to the diet. Increases were produced by adding niacin, lactose or milk powder to the basal diet; all these increases were prevented by adding sulfathalidine.

In experiments with rats a liver fraction was encountered which had a marked effect in relieving pteroylglutamic acid deficiency in rats which received a purified diet with sulfonamides. Its effectiveness appeared to be in excess of its pteroylglutamic acid content as measured by microbiological assay, even after treatment with acid, alkali, or a "conjugase" preparation had been used to liberate pteroylglutamic acid (155).

**Isolation of pteroylglutamic acid and related compounds.** Methods for the concentration of the "norite eluate factor" (pteroylglutamic acid) were described by the Wisconsin workers. In their first report (8) this factor was found to be relatively stable to acid and alkali; it could be adsorbed on activated charcoal and Lloyd's reagent; it was precipitated by phosphotungstic acid and by basic lead acetate and could be extracted from an acid solution by butanol. A later publication from the same laboratory (93) described additional methods of purification by adsorption on Superfiltrol. The factor was also concentrated from liver by adsorption and elution with norite and precipitation of the manganese salt with manganese chloride and ethanol (103).

The isolation of a highly active preparation of "folic acid" from spinach was reported by the Texas group (9) and was later described in more detail by the same group of workers (109, 156, 157). These workers employed repeated adsorption and elution from activated charcoal, precipitation with silver and lead, adsorption on Lloyd's reagent, chromatographic adsorption on alumina and precipitation of the free acid from cold acidic solutions. Since "folic acid" from spinach has never been crystallized it is not possible to establish its identity with pteroylglutamic acid. The activity of the material described in the first publication (9) had a "potency" of 40,000 as compared with a sample of a liver fraction B which was arbitrarily given a potency of 1. In a subsequent paper (156) a preparation with a "potency" of 137,000 was reported. While the activity of this preparation has not been given, a report (115) stated that synthetic pteroylglutamic acid gave a "potency" of approximately 137,000 when compared with a sample of "folic acid" of known potency obtained from spinach. Another communication (158) presented data showing that pteroylglutamic acid would have a "potency" of approximately 160,000. This data would indicate that the final product which was obtained from spinach and which had a potency of 137,000 was almost pure.

Two reports of the isolation of pure pteroylglutamic acid from liver have been made (10, 67). This compound was also isolated from a yeast concentrate which
had been enzymatically hydrolyzed (92). This yeast concentrate contained "B, conjugate" which was active in preventing anemia in chicks but which possessed little microbiological activity until after it had been hydrolyzed enzymatically. "Vitamin B, isolated from a hydrolyzed yeast concentrate was identical with pteroylglutamic acid obtained from liver.

Two different methods have been described for the isolation of pteroylglutamic acid from liver. One of these starts with whole liver in which the conjugates have been converted into free pteroylglutamic acid by autolysis (67, 159). The isolation process involved essentially the following steps: extraction with boiling water, adsorption on and elution from Amberlite 4R, adsorption and elution using activated charcoal, extraction of an aqueous solution of the free acid with butanol at pH 3 to 4, formation of a barium salt, extraction of barium salts with hot water, formation of a zinc salt and crystallization of the free acid from water. The other method (10, 160) employed as the starting material an 80 per cent-alcohol-insoluble fraction of an aqueous extract of liver. The fraction contained approximately 20 micrograms of pteroylglutamic acid per gram. The isolation process involved the following steps: adsorption and elution with norite, adsorption and elution with Superfiltrol, formation of a barium salt with barium chloride and methanol, esterification of the barium salt with 0.2N HCl methanol, extraction of the methyl ester from aqueous solution with butanol, chromatographic adsorption of the ester on Superfiltrol, and fractional precipitation of the ester from water and from methanol. The free acid obtained by hydrolysis of the ester was crystallized from hot aqueous solutions.

Properties of pteroylglutamic acid. Pteroylglutamic acid crystallizes as yellow spear shaped platelets. Its solubility (160) as the free acid is 10 micrograms per ml. at 0°C and more than 500 micrograms at 100°C. The sodium salt is much more soluble, having a solubility greater than 15 mgm. per ml. at 0°C. Pteroylglutamic acid has characteristic ultraviolet absorption spectra. In 0.1N NaOH it exhibits maxima at 257, 282, and 365 μ and corresponding E (1%)/1 cm values of 585, 570, 206. Another report (161) described the absorption spectrum at pH 11. This possessed maxima at the same points but with absorption coefficients which were slightly higher; 603, 600 and 213 at wavelengths of 256, 282, 365 μ.

The isolation of pteroyltriglutamic acid (85, 162) was accomplished by a method different from that used for the compound obtained from liver. The starting material was a cell-free filtrate obtained from aerobic fermentation of a diphtheroid-type organism. The method involved adsorption on charcoal, formation of a barium salt, esterification and extraction of the methyl ester from an aqueous solution with butanol and fractional precipitation of the ester from hot methanol. The methyl ester was dissolved in hot methanol and precipitated by cooling. In the presence of 0.05N sodium chloride the ester precipitated in a flocculent form while in the absence of electrolytes a gel was formed. After several such precipitations from methanol the ester was obtained in a microcrystalline form. The free acid was obtained in the pure state by converting the ester to the barium salt, removing extraneous pigments with Florosil and
precipitating the free acid at pH 2.8. The free acid could be crystallized from water containing electrolytes such as sodium or calcium chloride. In the absence of electrolytes gels were obtained. The pure ester was obtained by esterification of the free acid and crystallization of the ester from 0.05N sodium chloride in methanol.

While pteroylglutamic acid and its ester can be precipitated from water and methanol respectively in the absence of electrolytes, pteroyltriglutamic acid and its ester require the presence of electrolytes for their precipitation. The solubilities of pteroyltriglutamic acid in the presence of CaCl₂ at pH 2.8 are 3.0 mgm. per ml. at 80°C and 0.10 mgm. per ml. at 5°C.

A conjugate was isolated from yeast (70) and was later reported to contain seven glutamic acid residues (3). This compound was crystallized from 5 per cent sodium chloride. The use of electrolytes in crystallization of this compound was similar to that reported for the triglutamic acid derivative (162). The heptaglutamic acid conjugate possessed an activity for chicks which was proportional to its content of pteroylglutamic acid (70). Its microbiological activity was 0.3 to 0.6 per cent of that of pteroylglutamic acid by assay with *L. casei* and 0.2 per cent by assay with *S. fecalis* R (see table 2). The conjugate is as active on a molal basis as pteroylglutamic acid in promoting the growth of *Tetrahymena geleii* (108).

Degradation. The relationship between pteroyltriglutamic acid and pteroylglutamic acid was shown by anaerobic alkaline hydrolysis (163). The triglutamic acid derivative is active for *L. casei* but only slightly active for *S. fecalis* R (table 2). It was found that while aerobic alkaline hydrolysis produced rapid biological inactivation for both organisms, anaerobic hydrolysis produced only a slight decrease in the activity for *L. casei* and greatly increased the activity for *S. fecalis* R. The ratio of the activity for these two organisms approached that of pteroylglutamic acid isolated from liver. Two mols of alpha-amino acid nitrogen were liberated during anaerobic alkaline hydrolysis and the active compound which was formed was approximately half as active as pteroylglutamic acid by both *L. casei* and *S. fecalis* R assay. This compound was later identified as racemic pteroylglutamic acid.

Aerobic alkaline hydrolysis (163) of pteroyltriglutamic acid or racemic pteroylglutamic acid resulted in the formation of a fluorescent pigment and a diazotizable aromatic amine which could be estimated by the method of Bratton and Marshall (164). In the absence of oxygen no diazotizable amine or fluorescent pigment were produced by alkaline hydrolysis. The fluorescent pigment proved to be a dibasic acid having pKa values of 3.9 and 7.7. Elementary analysis suggested the empirical formula C₇H₁₆N₅O₄. Decarboxylation of the fluorescent dibasic pigment at 300° resulted in the liberation of approximately 1 mol of CO₂ and the formation of a fluorescent monobasic acid with a pKa of 8.0. Oxidation of the original dibasic acid with chlorine water, followed by hydrolysis with 0.1N HCl at 140°C yielded a compound which gave a positive test for guanidine. The formation of guanidine under such conditions constitutes evidence for a pyrimidine ring with an amino group in the 2-position (165). The fluorescent
dibasic acid showed characteristic absorption spectra in 0.1N NaOH with maxima at 253 and 365 m\(\mu\). The empirical formula C\(_7\)H\(_8\)N\(_6\)O\(_5\), the titration data, and formation of guanidine suggested a 2-amino purine or a 2-amino pteridine. The absorption spectra, however, eliminated the possibility of a purine because purines do not have absorption maxima above 300 m\(\mu\). Thus the available evidence pointed toward a 2-amino pteridine with an enolic and a carboxy group. With this evidence available, attempts were made to synthesize pteridine having these functional groups. The compound was identified as 2-amino-4-hydroxypteridine-6-carboxylic acid by comparison with the synthetic compound.

The monobasic fluorescent pigment produced by decarboxylation was identified as 2-amino-4-hydroxypteridine.

The structure of these two pteridines was established by the following series of reactions (166). Diethylmesoxalate was condensed with 2,4,5-triamino-6-hydroxypurimidine to yield isoxanthopterin carboxylic acid (II) (167). The structure of isoxanthopterin carboxylic acid (II) is that shown by formula II although it had not previously been definitely established whether the carboxyl group occupied the 6- or 7-position. On chlorination of isoxanthopterin carboxylic acid and subsequent reduction with hydrogen iodide one of the hydroxyl groups was removed to give compound IV which was identical with the dibasic fluorescent pigment (166). Presumably either the 4 or the 7 hydroxyl could have been removed by this procedure. The presence of the 4 hydroxyl in compound IV was shown in two ways. First decarboxylation of 2-amino-4-hydroxypteridine-6-carboxylic acid (IV) gave 2-amino-4-hydroxypteridine (V), the structure of which was established by its synthesis from glyoxal and 2,4,5-triamino-6-hydroxypurimidine (I). Its formation by this method demands a hydroxyl group in the 4 position. Second, the synthesis of 2-amino-4-hydroxypteridine-6-carboxylic acid was accomplished by condensation of 2,4,5-triamino-6-hydroxypurimidine (I) and ethyl-\(\beta,\beta\)-diethoxy-\(\alpha\)-bromo propionate. This reaction also establishes the presence of a hydroxyl group in the 4 position of the pteridine.

The final proof establishing the 6 position of the carboxyl group in compound IV was obtained by degrading 2-amino-4-hydroxy-6-methyl pteridine (VI) to give a compound identical with 2-amino-5-methyl-pyrazine (VII). The corresponding 7-methyl pteridine would have yielded 2-amino-6-methyl-pyrazine instead. The structure of 2-amino-4-hydroxy-6-methyl pteridine (VI) was established by oxidation with alkaline permanganate to give the corresponding 2-amino-4-hydroxypteridine-6-carboxylic acid (IV). These reactions are outlined in Fig. 2.

By hydrolysis with 0.5N sulfurous acid at room temperature pteroyltriglutamic acid was rapidly inactivated and gave an aromatic amine and a fluorescent pigment (168). This pigment reacted rapidly with typical aldehyde reagents such as hydroxylamine, phenylhydrazine and semicarbazide, indicating the presence of an aldehyde group. This fluorescent pigment did not possess a carboxyl group as evidenced by the fact that its distribution coefficient between water and buta-
Fig. 2. Chemical reactions leading to the establishment of the structural formula of the pteridine (IV) obtained by aerobic alkaline hydrolysis of *L. casei* factor.
nol was the same at pH 3.0 as at pH 7.0. When the pigment obtained by sul-
furous acid hydrolysis was treated anaerobically with dilute sodium hydroxide, 
approximately equal amounts of 2-amino-4-hydroxypteridine-6-carboxylic acid 
(IV) and a second pteridine, identified as 2-amino-4-hydroxy-6-methyl-
pteridine (VI), were formed. The latter compound could be oxidized with alka-
line potassium permanganate to yield 2-amino-4-hydroxypteridine-6-carboxylic 
acid (IV). The formation of approximately equal molal quantities of carboxy-
and methyl-derivatives from what is apparently an aldehyde probably involves 
a Cannizzaro type reaction although the mechanism of this reaction is obscure.

Prolonged aqueous hydrolysis of pteroyltriglutamic acid at pH 4 produced 
biological inactivation and yielded a compound which was crystallized and iden-
tified as 1-pyrollidonecarboxylic acid (168). On hydrolysis with alkali this 
yielded 1 (+)-glutamic acid which was estimated microbiologically.

The aromatic amine which was produced during sulfuric acid hydrolysis 
was isolated as the barium salt (168). This compound when diazotized and 
coupled with N(1-naphthyl)ethylenediamine dihydrochloride yielded a red 
pigment (164) which indicated a primary aromatic amine with a highly negative 
substituent group. The aromatic amine nitrogen as measured by the method 
of Bratton and Marshall (164) constituted approximately 25 per cent of the total 
nitrogen. The remaining 75 per cent of the nitrogen could be converted into 
alpha-amino-acid nitrogen by alkaline hydrolysis. From such hydrolysates 
the aromatic amine was isolated and identified as p-aminobenzoic acid. Micro-
biological assay of the hydrolysate indicated the presence of 3 mols of glutamic 
acid. The peptide linkage of the glutamic acid to p-aminobenzoic acid must 
involve the carboxyl group of the latter as a primary aromatic amine is required 
for reaction in the Bratton and Marshall test (164).

The diazotizable aromatic amine obtained by aerobic alkaline hydrolysis of 
racemic pteroylglutamic acid was found to contain 2.1 atoms of nitrogen for 
each atom of aromatic amino nitrogen (163). The distribution coefficient of 
this aromatic amine was greatly different from that of p-aminobenzoic acid. 
After hydrolysis with 2N sulfuric acid, 45 per cent of the total nitrogen appeared 
as alpha-amino-acid nitrogen, and the distribution coefficient of the aromatic 
amine became the same as that for p-aminobenzoic acid. The latter compound 
was isolated from the hydrolysate.

Evidence regarding the mode of linkage is furnished by the results of alkaline 
hydrolysis (163). The absence of fluorescence and of the free aromatic amine 
in the original pteroylglutamic acid, and the simultaneous appearance of these 
two during aerobic alkaline hydrolysis suggested that the pteridine is linked 
to the aromatic amine nitrogen. As hydrolysis proceeded the liberation of 
pteridine and aromatic amine appeared at approximately the same rate.

Reduction in acid solution either catalytically or with zinc dust yielded the 
aromatic amine and a reduced pteridine (168). After reoxidation with manga-
nese dioxide the pteridine obtained by zinc reduction was identified as 2-amino-4-
hydroxy-6-methylpteridine, the structure of which has already been estab-
lished.
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The foregoing evidence indicated the following conclusions regarding the structure of pteroylglutamic acid.

1. Aerobic alkaline hydrolysis, sulfurous acid cleavage, and chemical or catalytic reduction each yielded a pteridine and a primary aromatic amine. This indicated the linkage of the pteridine to the nitrogen of the aromatic amine.

2. The aromatic amine formed during sulfurous acid cleavage of pteroylglutamic acid was a tetrapeptide, p-aminobenzoyldiglutamylglutamic acid. The aromatic amine from pteroylglutamic acid was p-aminobenzoylglutamic acid.

3. There was a single carbon atom linkage between the pteridine and the aromatic amine. This was indicated by the fact that only pteridines with a single carbon atom side chain were obtained and that no other two-carbon fragments could be detected in the two degradation reaction products. The evidence also indicated that this single carbon atom is present in a methylene link. If it were present as a C=O group in an amide linkage the cleavage would be hydrolytic and would not require oxygen. The formation of 2-amino-4-hydroxy-6-methylpteridine also constituted evidence for the methylene linkage.

Synthesis. The final proof of the structure was obtained by synthesis of pteroylglutamic acid by different methods. One of these (101) involved the simultaneous condensation of 2-4-5-triamino-6-hydroxypyrimidine (I), p-aminobenzoylglutamic acid (IX) and α,β-dibromopropionaldehyde in aqueous solution. The other (169) involved the reaction of α,β-dibromopropionaldehyde with pyridine, 2,4,5-triamino-6-hydroxypyrimidine (I) and potassium iodide to yield N[(2-amino-4-hydroxy-6-pteridyl)methyl] pyridinium iodide (X). This was then treated with p-aminobenzoylglutamic acid (IX) in ethylene glycol to yield pteroylglutamic acid. The position of the methyl pyridinium group on the 6 position of the pteridine was established by oxidation with alkaline permanganate to yield 2-amino-4-hydroxypteridine-6-carboxylic acid (IV). These two methods of synthesis are schematically outlined in Fig. 3

Pteroyic acid was obtained by the corresponding reaction (101) except that p-aminobenzoic acid was used instead of p-aminobenzoylglutamic acid.

Pteroylglutamic acid in the nutrition of mice. Indications that folic acid was required by mice on a purified diet containing sulfasuxidine were reported (170). The “lactation performance” of mice on purified diets as measured by the percentage and size of litters weaned was increased by adding a concentrate containing pteroylglutamic acid (171, 172).

Dogs. Observations with dogs (173) and pigs (174) indicated that an unidentified B complex factor or factors present in liver and yeast, were needed as a supplement with purified diets. It seems probable that the liver and yeast extracts used in these investigations supplied pteroylglutamic acid. Pteroylglutamic acid was reported to improve the response to niacin in dogs on purified diets deficient in niacin (175).

Guinea pigs. A series of studies with guinea pigs on purified diets indicated that three unidentified dietary factors were required, two of which were found present in linseed oil meal. It was later shown that pteroylglutamic acid could
Fig. 3. Two syntheses of pteroylglutamic acid.
replace one of the factors (176). The deficiency was described as being characterized by a rapid decline in weight, lethargy, salivation, terminal convulsions and death. The daily administration of 6.5 micrograms of either pteroylglutamic acid or pteroyltriglutamic acid prevented the deficiency.

**Nutrition of insects.** Larvae of the mosquito *Aedes aegyptii* were found to need concentrates of pteroylglutamic acid for pupation; xanthopterin or thymine were ineffective. Pteroylglutamic acid was also found to promote growth and to increase survival rate (177). It was found that the larvae of the flour moth and the meal worm needed pteroylglutamic acid for growth (178). Growth of the larvae of a carpet beetle was increased by concentrates of pteroylglutamic acid (179).

**Mink.** The effects of pteroylglutamic acid deficiency in mink were found to include loss of body weight, diarrhea, irritability, general weakness, anorexia and leukopenia. The deficiency responded to administration of pteroylglutamic acid. There was some indication that an unidentified factor in liver was also needed (180).

**Pigs.** It was suggested that pteroylglutamic acid deficiency in pigs might be associated with the development of normocytic anemia, although no actual test with pteroylglutamic acid was carried out (181). The pigs from sows fed a supplementary “folic acid concentrate” were thriftier and more vigorous than were pigs from sows fed the basal diet of corn and soy bean meal supplemented with vitamins, not including pteroylglutamic acid, and minerals (182). It was reported that pteroylglutamic acid “may have helped slightly with hemo-globin formation” in pigs on a purified diet (183). Other observations (174) were mentioned above.

**The enzymic liberation of pteroylglutamic acid from its “conjugated” forms.** The effect of enzymic action in liberating folic acid from tissues was indicated by an observation that the apparent “folic acid” content of such materials could be increased several-fold by treatment with a crude enzyme-containing preparation (“taka-diastase”) (184). It was found possible to prepare a dialyzable fraction from yeast which was active for the “vitamin B₁₂” deficient chick but which had relatively little potency in stimulating the growth of *L. casei* (92) until the concentrate was subjected to an undescribed “enzymatic digestion” which liberated “vitamin B₁₂” in a microbiologically active form.

Almost at the same time, it was noted that yeast was “rich in the substances which give rise to folic acid when incubated with fresh liver” (30). A crude enzymically-active preparation was made from rat liver (36) by extraction with phosphate buffer, fractional precipitation with ammonium sulfate, and dialysis. The preparation liberated the *S. lactis* R-stimulating factor at pH 7 from cell-free water-soluble fractions. These fractions had previously been extracted from natural materials with the aid of “taka-diastase” which thus had brought pteroylglutamic acid conjugates into solution without splitting them. Hence taka-diastase did not supply appreciable quantities of the “folic-acid-liberating” enzyme system. This observation indicated a degree of specificity on the part of the enzyme concentrate prepared from rat liver. The name “vitamin B₁₂
conjugase" was applied to the enzyme system (185). Hog kidney, liver, small intestine and beef liver were reported to be rich sources; it also occurred in sweet almonds. The optimum pH of the hog kidney preparation was 4.5, while that for almond was 7.0. Hog kidney was used as a source of the enzyme in a method for the assay of "vitamin B₉ conjugate" (186). Samples were incubated for 16 hours at pH 4.5 and 45°. The values obtained after enzymatic treatment in many cases showed close agreement with the values obtained by biological assay with chicks on a purified diet (68). An exception was observed in the case of certain liver extracts and plant extracts. The presence of inhibitors in the plant extracts was suggested, or, as an alternative, the presence of compounds which were active for the chick but inactive for L. casei even after treatment with the enzyme.

Chicken pancreas (187) was found to be an excellent source. An increase in potency of more than one-thousand fold was reported to occur upon concentrating the enzyme from chicken pancreas extract by adsorption and precipitation techniques. The optimum pH for the action of the enzyme was between 7 and 8; a considerable loss of activity was observed on dialysis. Further studies (158) described modifications in the procedure for concentrating the enzyme. The pancreatic tissue was ground and allowed to autolyze at pH 8. A fraction was then separated which precipitated at between 40 and 80 per cent saturation of ammonium sulfate. The precipitate was dialyzed, redissolved, reprecipitated with cold alcohol, redissolved in buffer and reprecipitated with ammonium sulfate. The procedure resulted in an approximately 3000-fold concentration of activity. The concentrated preparation was activated by calcium, the optimal concentration being 0.01M. Optimum pH and temperature were found to be 7.8 and 32°.

The Michaelis constant for conjugase preparations from rat liver, potatoes and chicken pancreas was measured (188).

The production of "vitamin B₉ activity" in several hundred different microorganisms was measured by L. casei assay. Various commercial enzymic preparations were tested for their activity in liberating pteroylglutamic acid from its conjugates but none of the preparations were very effective. Chicken pancreas was used as a source of the enzyme (189).

A study was made of the comparative potencies of various tissues in liberating pteroylglutamic acid from a concentrated preparation of pteroylheptaglutamic acid (vitamin B₉ conjugate) (190). Chicken and turkey pancreas were the most potent tissues; their optimum activity was at a pH of about 7.0 while other tissues; including rat, mouse, hog and guinea pig pancreas, chicken and hog liver and hog kidney had their optimum at a pH of about 4.5. Hog kidney was used as a source of the "conjugase" enzyme system for further studies. The optimum temperature for the action of the enzyme was found to be 45° to 48°. The presence of an inhibitor in yeast extract was demonstrated. Loss of activity occurred during attempts to concentrate the conjugase; the addition of calcium failed to restore the loss. A unit of activity for the enzyme was proposed and defined.

The p-aminobenzoyl-polyglutamic-acid polypeptide isolated from yeast (191)
was found under certain circumstances to inhibit "vitamin B, conjugase" preparations obtained from chicken pancreas and rat liver (Totter, J. R. Cited in (192)).

Clinical effects of pteroylglutamic acid. It is beyond the scope of this review to discuss the extensive literature of recent years regarding the erythrocyte maturation factor present in liver and the extrinsic and intrinsic factors which have been postulated to be concerned in its formation. Mention must be made, however, of the baffling circumstances that simultaneously relate and contrast pteroylglutamic acid and the erythrocyte maturation factor. Both are present in liver, and both will produce hematologic remission of addisonian pernicious anemia. However, the processes commercially used in the refinement of concentrated solutions of the erythrocyte maturation factor do not result in the concentration of pteroylglutamic acid, in fact the original pteroylglutamic acid content of crude liver extracts is largely diverted into side fractions during the process. Experimental animals, even when they are acutely deficient in pteroylglutamic acid, do not respond to preparations of the erythrocyte maturation factor. In the treatment of pernicious anemia, the erythrocyte maturation factor is far more effective when injected than when fed to pernicious anemia patients, while pteroylglutamic acid is approximately equally effective by either route of administration. Differentiation of pteroylglutamic acid from the "extrinsic factor" appears to be established by the activity of pteroylglutamic acid when fed or injected in producing hemopoietic remission of pernicious anemia without the addition of normal human gastric juice.

The results of various clinical experiments, most of which did not permit of definite conclusions and which were made with crude materials or natural concentrates, were published prior to the availability of pteroylglutamic acid. The investigations of Wills were mentioned on p. 54; these findings served to differentiate the erythrocyte maturation factor from an unidentified factor in yeast and liver which was effective against tropical macrocytic anemia and against a corresponding anemia in monkeys. Recently this question was reopened (193) in an article which drew attention to preceding observations (194, 195, 196, 197, 198, 199, 200) that certain macrocytic anemias usually associated with pregnancy and encountered both in the tropics and the temperate zone did not respond to the injection of liver extracts which were effective against pernicious anemia but these anemias responded to the oral administration of liver or autolyzed yeast. Three cases with histories of "striking dietary inadequacy" were found to respond to oral liver extract after no response had occurred to smaller volumes of injected liver extract.

Five patients with pernicious anemia were found to respond to comparatively large doses of brewers' yeast 1 to 2 grams per kilo of body weight daily without the addition of gastric juice (201). One may speculate that this response was probably due to pteroylglutamic acid.

A concentrate of "vitamin B," from yeast, standardized by chick assay, was fed to ten patients with refractory macrocytic anemias. A dosage rate corresponding to 0.6 mgm. of pteroylglutamic acid daily was used for the first week,
and it was increased to 1.5 mgm. daily for the next 3 weeks. With the exception of an increase in the hematocrit reading, no significant changes were obtained in the blood picture (202).

Negative results were obtained in the treatment of 2 cases of pernicious anemia with pteroylglutamic acid 3.6 or 2.3 mgm. daily by mouth for 10 days (203). This may be contrasted with the subsequent report of a single case which responded to pteroylglutamic acid, 3 mgm. daily, by injection (204).

As a result of the preceding and other studies (16, 193, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214) it became evident by 1945 that there existed a nutritional macrocytic anemia often associated with pregnancy, which responded to the feeding of crude liver extracts, yeast or yeast extracts, but which did not respond to the injection of potent sources of the anti-pernicious-anemia factor of liver extracts. The anemia was characterized in some cases (215) by a history of marked dietary inadequacy especially with respect to meat; the presence of gastric hydrochloric acid; the absence of neural manifestations; and the presence of a megaloblastic bone marrow. Maintenance therapy with liver was not required. A similar condition was encountered which responded to the injection of concentrated liver extracts (193, 194, 216, 217, 218, 219); the differentiation of this from the preceding type of anemia appeared to be possible only on the basis of response to such extracts. The time was now ripe for the appearance of a new therapeutic substance. This substance proved to be pteroylglutamic acid.

In the summer of 1945, synthetic pteroylglutamic acid was made available in fairly large quantities for clinical experiments. Results were soon obtained and reported in the literature; it was apparent that the use of adequate dosage schedules led to the establishment of positive responses to pteroylglutamic acid in the treatment of anemias which were accompanied by megaloblastic erythropoiesis.

**Addisonian pernicious anemia.** Descriptions of the response of this disease to pteroylglutamic acid first appeared in December, 1945 (22, 220).

The effects of pteroylglutamic acid on 14 cases of macrocytic anemia in relapse were described (22). Five of these cases were classified as addisonian pernicious anemia, five as nutritional macrocytic anemia, and two as indeterminate. The patients received pteroylglutamic acid, 20 mgm. to 50 mgm. injected or 100 mgm. by mouth daily. One patient received 150 mgm. by mouth. A feeling of subjective improvement occurred between the third and fifth days and was accompanied by an increase in appetite, which was in some cases associated with "remarkable weight gains", in most patients. The initial red blood cell count varied from 1.61 million to 2.97 million cells per cu. mm. Except in the case of one patient who did not respond, the final red cell count at 18 to 55 days varied from 2.92 to 4.29 million cells per cu. mm. Oral administration produced greater reticulocyte responses and more rapid regeneration of blood than did parenteral administration. However, the oral dosage was usually 5 times as great as the parenteral. Increases were also noted in per cent hemoglobin and in total white cell count. Reticulocyte crises were observed in from 3 to 10 days.
The administration of pteroylglutamic acid was continued for periods up to 30 days and the red cell count and the per cent hemoglobin continued to rise after the treatment was discontinued.

In the other study (220) two patients with pernicious anemia were studied. The first patient with pernicious anemia was given 100 mgm. of pteroylglutamic acid orally each day for 10 days. Her initial red blood cell level was approximately 1.2 million cells per cu. mm. On the third day of therapy she experienced a feeling of well-being and increased appetite and on the next day her reticulocytes began to rise and a peak value of 40 per cent was reached on the seventh day. Her red blood cells began to increase in number on about the seventh day and the count rose rapidly until a level slightly over 3 million was reached. The second patient responded in a similar manner.

The use of pteroylglutamic acid in a case of untreated addisonian pernicious anemia was described (221). The patient received 2.0 mg. of pteroylglutamic acid intravenously daily for 20 consecutive days. The red cell count before treatment was 1,490,000 per cu. mm. with 5.8 grams of hemoglobin. Microscopic examination of the bone marrow showed a picture typical of a deficiency of the maturation factor. After treatment “daily bone marrow examinations demonstrated a gradual megaloblastic maturation with the marrow picture approaching normal on the tenth day of therapy.” The maximum reticulocyte response (26.8 per cent) was obtained on the fiftieth day of therapy, after a total cumulative dosage of 30 mgm. to see if a secondary reticulocyte rise could be induced. This was not observed. On the fortieth day of therapy the red cells reached 4,000,000 per cu. mm. and the hemoglobin 13.0 grams. There had been a definite corresponding rise in total white cells and platelets. The same publication described the effects of pteroylglutamic acid in three patients sensitive to liver extract. In each instance, the local reaction to the injection of liver extracts was extremely marked, and in no instance was there any reaction to pteroylglutamic acid greater than to normal saline.

In another report (222) hemopoietic responses to pteroylglutamic acid in 5 cases of pernicious anemia were described after daily dosage with 20 mgm. of pteroylglutamic acid intravenously or 100 mgm. by mouth. Increases in erythrocyte counts and hemoglobin levels were observed, together with reticulocyte crises ranging from 6.4 to 14.8 per cent. Positive responses to pteroylglutamic acid in the treatment of 4 cases of pernicious anemia were described (223). Results similar to the preceding were noted (23), and it was found that one patient with pernicious anemia had neurologic changes which improved with pteroylglutamic acid therapy, although complete return to normal had not been obtained at the time of writing.

The responses of 6 patients with addisonian pernicious anemia to pteroylglutamic acid were described (224). Clinical improvement and hematological remission were noted in all cases. In one case muscular weakness and loss of vibratory sense of both lower extremities were observed; these findings were unchanged after 79 days of treatment with pteroylglutamic acid, 25 mgm. to 100 mgm. daily.
Other reports (225, 226, 227) described hematological responses to pteroylglutamic acid in pernicious anemia patients. Studies were reported (227) on the response of various macrocytic anemias. Macrocytosis alone did not necessarily imply either megaloblastic erythropoiesis in the marrow nor responsiveness to pteroylglutamic acid. It was considered justifiable to regard megaloblastic erythropoiesis as the morphologic expression of pteroylglutamic acid deficiency. The authors studied 11 cases of macrocytic anemia treated with pteroylglutamic acid. Five of these were pernicious anemia cases which responded promptly. The remaining six cases, all of which showed normoblastic marrow patterns, did not respond; these included four cases of portal cirrhosis, one of acute infectious hepatitis (Weil's disease) and one of subacute lymphatic leukemia.

**Pteroyltriglutamic acid.** A patient with pernicious anemia in relapse was treated with pteroyltriglutamic acid, 3 mgm. daily, by intramuscular injection for 11 days. A submaximal hemopoietic response was observed, accompanied by subjective improvement (204). It may be presumed that a more marked response could have been obtained with a higher dosage.

**Thymine.** Observations of the growth-promoting effects of this substance on *L. casei* and *S. fecalis* R (121, 103, 105) were followed by studies of its effect in pernicious anemia. Large amounts were necessary to produce an effect; the administration of 1 gram or less gave no response in pernicious anemia, sprue or nutritional macrocytic anemia (228). One case of pernicious anemia responded to 6 grams daily by mouth for 14 days; three cases to doses up to 3.4 grams daily for 11 days (229) and six cases to doses of 4.5 to 10.2 grams daily (230). The necessary amounts appeared to be in the neighborhood of 1000 times as great as the adequate dosage of pteroylglutamic acid. A similar ratio of effective dosage between thymine and pteroylglutamic acid was observed with the lactic acid bacteria (see p. 65).

**Combined system disease.** By the summer of 1946 it had been well established that pteroylglutamic acid would consistently produce a prompt and satisfactory hemopoietic response in addisonian pernicious anemia. A long period of study was necessary to compare the effects of liver extract and pteroylglutamic acid in preventing the onset of neurological signs and symptoms in pernicious anemia. The results of a 12-month investigation of this point were reported (231). Twenty-six patients were studied, 21 of whom had pernicious anemia which had been controlled for from 2 to 17 years by injections of liver extract. Three had pernicious anemia in relapse and two had sprue which had been poorly controlled by liver extract. Liver therapy was discontinued and the patients were treated with 70 to 105 mgm. pteroylglutamic acid per week in divided doses given orally for 10 to 12 months. Most of the patients noted an increase in appetite and weight. In two instances there were moderate increases in the hematological values after 5 to 8 months which increased until there was evidence of combined system disease. The dosage of pteroylglutamic acid was increased without signs of improvement, following which refined liver extract, 5 cc. daily,
was administered and neurological improvement was reported to be observed in
10 days.
In another study (232) the following data were obtained regarding the effect
of pteroylglutamic acid on 14 cases of pernicious anemia.

<table>
<thead>
<tr>
<th>GLOSSITIS</th>
<th>PARESTHESIA</th>
<th>COMBINED SCLEROSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Improvement</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Temporary improvement</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>No improvement</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>New manifestations</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

The effect of pteroylglutamic acid on pernicious anemia and combined system
disease was studied in a number of patients (233). Three patients with neuro-
logical involvement received pteroylglutamic acid, 25 to 50 mgm. by mouth or
20 mgm. intramuscularly daily. The patients showed hematological responses
but their neurological symptoms continued to progress. Administration of
liver extract resulted in improvement in the signs of nervous system changes.
Other pernicious anemia patients in relapse received 0.5 unit of liver extract
with 5 to 10 mgm. of pteroylglutamic acid daily and showed reticulocyte re-
sponses which were greater than those anticipated as a result of previous expe-
rience with liver alone.

Rapidly progressive neurological relapse was observed in a patient with per-
nicious anemia who had been maintained on pteroylglutamic acid for 12 weeks.
The diet during this period was poor. The patient subsequently improved
during treatment with liver extract. The patient was one of a group of 47 pa-
tients with pernicious anemia who were maintained with pteroylglutamic acid
for periods up to one year. Only two others showed neurological relapse.
Both of these had a poor dietary history. They showed mild symptoms which
responded readily to liver extract (234).

These preliminary studies indicated that the erythrocyte maturation factor,
or some unidentified substance which accompanies it in therapeutic amounts in
refined liver extract, is needed for the prevention of neurological signs and symp-
toms in certain cases of combined system disease and that pteroylglutamic
acid is not consistently effective in this regard. It would be of interest to study
the effects of pteroylglutamic acid upon the incidence of combined system disease
when administered together with large amounts of the known B complex vita-
mins.

Possible relation of pteroylglutamic acid to the biochemical defect in pernicious
anemia. A disturbance in the utilization of pteroylglutamic acid appears to
occur in pernicious anemia. This is evidenced by the following considerations:

a. In contrast to certain other anemias, pernicious anemia has never been associated with an obviously incomplete diet.

b. Dosage with pteroylglutamic acid, usually in amounts which are greater than those occurring in ordinary diets, will produce a hemopoietic response in pernicious anemia.

c. Certain cases of pernicious anemia do not respond to crude concentrates of pteroylheptaglutamic acid. This finding is discussed below.

Preliminary publications speculated upon the possibility that a basic defect in pernicious anemia is a failure to liberate pteroylglutamic acid from its conjugates (235, 236). It was observed (236) that when pteroylheptaglutamic acid (vitamin B\(_7\) conjugate) was administered to normal individuals there was increased urinary excretion of pteroylglutamic acid. However, no such increase was observed when pteroylheptaglutamic acid was administered to pernicious anemia patients (235, 236). It was therefore portulated that in these patients a pteroylglutamic acid deficiency resulted from the imperfect utilization of conjugated forms of pteroylglutamic acid and that as a result the supply of pteroylglutamic acid was insufficient for the maintenance of the hemopoietic mechanism. Three patients with pernicious anemia and one with macrocytic anemia following gastrectomy showed no evidence of a therapeutic effect from the administration of pteroylheptaglutamic acid, equivalent to 2.3 to 4 mgm. of pteroylglutamic acid daily for 8 to 12 days. When equivalent amounts of pteroylglutamic acid were substituted for the conjugate all of the patients showed significant clinical and hematologic responses (235). It was reported (236) that the daily administration of 1 mgm. of "yeast conjugate", presumably pteroylheptaglutamic acid, for 10 days to a patient with pernicious anemia in relapse produced no response. During a second period of 11 days, 100 cc. of normal human gastric juice was administered with the yeast conjugate, again without response. The patient then responded submaximally to a daily dose of 0.35 mgm. of pteroylglutamic acid. A second patient received intramuscular injections of 2.5 mgm. of yeast conjugate daily for 12 days, followed by a single injection of 30 mgm. of conjugate, without responding. After a further 12 days, administration of pteroylglutamic acid causes a theoretically maximal reticulocyte response. In two patients with pernicious anemia, injection of concentrated liver extract containing insignificant amounts of pteroylglutamic acid doubled the urinary excretion of pteroylglutamic acid. This observation fitted the interpretation that a constituent of liver extract might be concerned with the activation of a "conjugase system", or with the removal of an inhibitor of such a system (237) but it should be noted that the urinary excretion of pteroylglutamic acid on a normal diet is extremely small both in normal subjects and in pernicious anemia patients, so that doubling the urinary excretion of pteroylglutamic acid is quantitatively only a small increase.

In another report (238) earlier findings (235) were extended. The heptaglutamate was given orally in the form of a concentrate to nine cases of pernicious anemia in relapse, three in remission induced by liver extract, two with macro-
cytic anemia following gastrectomy, and six healthy subjects, without effect on the anemias, all of which subsequently responded to pteroylglutamic acid. No pteroylheptaglutamic acid was found present in the urine in any of the experiments. The patients excreted variable amounts of pteroylglutamic acid following its administration while the amounts excreted by the normal subjects were “considerably higher and more constant”. After the administration of pteroylheptaglutamic acid the patients with one exception showed no significant increase in excretion of pteroylglutamic acid, but when pteroylheptaglutamic acid was administered to the patients in remission induced by liver extract, an excretion of pteroylglutamic acid in amounts similar to those observed with healthy subjects was found. This suggested that “the principle of liver active in pernicious anemia may be concerned with the conversion of the conjugated vitamin to the free form”.

More detailed findings were subsequently reported (239) and the earlier postulations regarding the function of the erythrocyte maturation factor were modified in the light of these results. Emphasis was laid upon the role of “conjugase inhibitors”, as present in certain yeast concentrates, in modifying the utilization of “hexaglutamyl conjugate” (pteroylheptaglutamic acid) by pernicious anemia patients. The urinary excretion of pteroylglutamic acid after the administration of conjugate with “large amounts of inhibitor” was much less in pernicious anemia patients than in normal subjects. Two patients, one in relapse and one in partial remission, were treated with conjugate plus liver extract without markedly increasing the urinary pteroylglutamic acid excretion over the levels observed when the conjugate was given alone. In contrast, three patients in remission who received the conjugate excreted pteroylglutamic acid in amounts approximately equal to those observed in normal subjects. It was concluded that the diminished ability of pernicious anemia patients to utilize the conjugate was not absolute, varied in different patients, and was partly dependent upon a conjugase inhibitor present in natural materials. The effect of the inhibitor was studied in normal persons (240), and its administration was found markedly to reduce the urinary excretion of pteroylglutamic acid following the feeding of conjugate, 4 mgm. daily. Attention was drawn to the high inhibitor content of liver, yeast and spinach. The conjugate was not found present in the urine in any of the experiments.

Others have also pointed out that certain pernicious anemia patients are able to utilize concentrates of the conjugates (192). It is difficult in the present state of knowledge to generalize regarding the biochemical defects in pernicious anemia. It appears to be evident that there is an abnormality in the utilization of pteroylglutamic acid. The variability in the findings may be due to variations in the extent and type of the biochemical lesions in the patients.

Another basis for possible speculation lies in some observations of the excretion of xanthopterin (47). It was reported that the normal daily excretion of xanthopterin in the urine was increased by approximately 50 per cent in untreated pernicious anemia. The excretion dropped to normal in 11 of 13 patients after remission had been brought about by liver extract. If pteroyl-
glutamic acid is broken down to xanthopterin and is excreted as such in the urine, these observations might imply that there is an increased rate of breakdown of pteroylglutamic acid in pernicious anemia. However, two other patients in this study had extremely low urinary xanthopterin values which increased after treatment with liver extract.

Sprue. The successful treatment of sprue with pteroylglutamic acid was first reported in November 1945 (241). Two patients were treated daily with 15 mgm. of pteroylglutamic acid by intramuscular injection. The symptoms of glossitis disappeared after 4 days treatment in the first case. After nine days the reticulocytes reached a peak of 15.3 per cent and a marked increase in thrombocytes was noted. The red cell count rose from 1.56 to 3.88 cells per cu. mm. and the per cent hemoglobin rose from 6.0 to 9.5. These changes were accompanied by marked general betterment, including regeneration of the lingual papillae, subsidence of the diarrhea and considerable gain in weight. The tolerance curves for oral glucose and vitamin A became more nearly normal. The second case received similar treatment and showed a reticulocyte crisis on the 4th day accompanied by subjective improvement.

In another report (220) 20 mgm. of pteroylglutamic acid was injected intravenously each day for 10 days to a patient with sprue. Following the initial ten-day period 40 mg, was injected every other day for an additional two weeks. A reticulocyte peak of 30.2 per cent was obtained on the seventh day although the initial red count was 2.6 million cells, so that the reticulocyte response was greater than was anticipated. The patient gained 8 pounds within 3 1/2 weeks and the oral glucose tolerance test showed much better absorption.

Three cases of "tropical" sprue were treated with 200 mgm. of pteroylglutamic acid daily by mouth (242). Subjective improvement was noted after 3 or 4 days. The erythrocyte count was 1.15 to 2.16 million cells per cu. mm. on the 10th day. Reticulocyte crisis occurred in 6 to 7 days with peak values of 17 per cent to 22 per cent. Gains in strength, vigor and appetite were noted.

Further laboratory findings regarding patients with sprue were reported (243). Three patients were studied who fulfilled all of the criteria necessary for the diagnosis of sprue including glossitis, diarrhea with increased fat content of the stools, marked loss of weight, pigmentation of the skin, macrocytic anemia, moderate leucopenia, impairment of absorption as indicated by a flat oral glucose tolerance curve with a normal intravenous tolerance curve, a flat vitamin A tolerance curve and a very low serum carotene content, and a characteristic gastrointestinal pattern on x-ray examination. Free hydrochloric acid was present in the gastric juice of each. Sternal marrow of two of the patients was examined and found to be typical of that seen in untreated sprue and pernicious anemia. After a preliminary period of observation these patients received daily intramuscular injections of 15 mgm. of pteroylglutamic acid as the sole therapeutic agent. In all three cases prompt hematological and clinical improvement was noted including disappearance of glossitis within 3 or 4 days, regeneration of the lingual papillae, an improved sense of well-being, subsidence of diarrhea, improvement in appetite and gain in weight. One patient gained 26 pounds.
within 6 weeks following the institution of treatment with pteroylglutamic acid. Reticulocyte crises were noted in 6 to 8 days together with increases in the number of platelets, the number of white blood cells, the number of red blood cells and the percentage of hemoglobin. Examination of aspirated sternal marrow after treatment showed the disappearance of the more primitive red blood cells and return of the white cell series to normal proportions.

Other reports with sprue have indicated comparable findings (23).

The effect of pteroylglutamic acid on plasma tocopherol levels in sprue was reported (244). The level gradually decreased to a very low value in one patient during a relapse following the withholding of pteroylglutamic acid therapy and increased following readministration of pteroylglutamic acid. During relapse it was observed that the patient showed a maximum rise in serum concentration of tocopherols of 0.09 mgm. per 100 cc. after an oral dose of 600 mg. of mixed tocopherols in contrast to an average peak of 0.37 mgm. in seven healthy adults after a similar dose. Attention was drawn to findings elsewhere that muscle tissue at autopsy from patients with sprue showed pigment resembling that seen in vitamin E deficient animals and that tocopherol deficiency has been observed in rats on purified diets containing sulfonamides.

Findings regarding the metabolism of pteroylglutamic acid and its conjugates were obtained in studies with two sprue patients (192). The first patient had been treated irregularly and inadequately with liver extract. He responded promptly to intramuscular injections of pteroyltriglutamic acid, 4.9 mgm. daily. After 10 days, concentrated liver extract was also administered, and the patient's recovery continued uneventfully. Microbiological assays of the urine showed that the amount of pteroylglutamic acid excreted daily before the initiation of treatment was so low as to be undetectable. Following the administration of pteroyltriglutamic acid, the urinary excretion of pteroylglutamic acid rose to a maximum of 3.66 mgm. on the sixth day, then declined to 1.34 mgm. on the tenth day. Upon starting liver extract which contained insignificant amounts of pteroylglutamic acid, the excretion rose to the remarkably high level of over 7 mgs. of pteroylglutamic acid daily for two days, in spite of the fact that the daily intake of pteroyltriglutamic acid corresponded to only 3.1 mgm. of pteroylglutamic acid. This finding indicated that some factor in liver extract had a marked effect on pteroylglutamic acid metabolism in sprue. The second patient was treated orally with a concentrate of pteroylheptaglutamic acid containing 8.4 mgm. of pteroylheptaglutamic acid and about 0.3 mgm. of free pteroylglutamic acid per daily dose. Remission promptly occurred with urinary excretion of only small amounts of pteroylglutamic acid. The excretion rose to a maximum of 0.32 mgm. on the tenth day, at which point the injection of liver extract was started, following which the urinary excretion continued to increase to a maximum of 1.5 mgm. Treatment of the urine of either patient with "conjugase" indicated the absence of conjugates. The number of cases is so small that the results until confirmed should be treated with reservation in spite of their great biochemical interest.

A patient with sprue was treated with 5 mgm. of pteroyltriglutamic acid twice
daily by intramuscular injection. A total quantity of 83 mgm. was given over a period of 9 days. A reticulocyte peak of 38 per cent occurred on the fifth day of therapy and was accompanied by a rise in erythrocyte count and in hemoglobin and by clinical improvement (245).

Thymine in sprue. Clinical and hematological improvement were reported in four patients with sprue who received 15 grams of thymine daily (246). Reticulocyte peaks were reached on the eighth or ninth day, and were followed by increase in erythrocytes and hemoglobin, subjective improvement and a return of the stools toward normal. The authors observed that the response was less dramatic than that observed with pteroylglutamic acid.

Nutritional macrocytic anemia. This condition was discussed and reviewed (17). In December 1945 (22), a consistent effect of pteroylglutamic acid in producing remission of 9 cases of nutritional macrocytic anemia was described. The responses were similar to those described for pernicious anemia in the same article (see p. 82). Additional details were given in a later publication (222). Similar results were obtained elsewhere (23). “Nutritional macrocytic anemia” occurring in a patient who had previously responded to pteroylglutamic acid and who had subsequently relapsed, was found to respond to pteroylheptaglutamic acid, 14 mg. daily for 9 days (247). The clinical improvement was rapid and was comparable to that observed with pteroylglutamic acid. A case of nutritional macrocytic anemia did not respond to pteroic acid, 5 mgm. by intramuscular injection daily for 8 days. The patient subsequently responded to dosage with pteroylglutamic acid, 5 mgm. daily.

Macrocytic anemia of pregnancy. A patient was described as having a red blood cell count of 1.1 to 1.2 million red blood cells per cu. mm. 18 days after parturition. She received 20 mgm. of pteroylglutamic acid intramuscularly for each of 10 days. Her subjective improvement was marked on the third day, and a peak value of 48 per cent reticulocytes was reached on the seventh day. The red cell count rose quite rapidly (220). Another report (24) described a response to pteroylglutamic acid in what was presumably a case of macrocytic anemia of pregnancy.

Megaloblastic anemia in infancy. A series of studies (248, 249, 250) described this condition as being characterized by:

1. Normochromic anemia, usually but not invariably macrocytic
2. A tendency toward leukopenia and neutropenia
3. A diminution of platelets, often associated with an increased bleeding tendency
4. A megaloblastic bone marrow pattern resembling or identical with the pattern seen in pernicious anemia in relapse
5. A frequent incidence of splenomegaly; an evidence of infection usually present; a histamine refractory achlorhydria present but reversible.

The treatment of 29 patients was described, 12 of these received pteroylglutamic acid either synthetic or in the form of a natural concentrate prepared by eluting a charcoal adsorbate of liver extract. The dosage rate was 5 to 20 mgm. per day for 8 days to 3 weeks. Three of the patients died with severe
infections. The remaining 9 responded, showing reticulocyte peaks, a return of the bone marrow pattern to normal, and increases in hemoglobin and red cell counts. Increases in the platelet counts were noted in blood smears at around the ninth day. The effect of pteroylglutamic acid was stated to be indistinguishable from that produced by liver extract and no relapses were observed in follow-up studies which lasted up to 10 months. The etiology of the condition, which was commonly associated with coughs and coryza, was indefinite. Of interest is the observation that one child had taken nothing but goats’ milk for four months. This recalls the early observations with rats (44, 46). Another early observation (251) described the response of “goats’ milk anemia” in infants to liver extract and to yeast. No response was obtained to iron.

Celiac disease. The occurrence of macrocytic anemia in celiac patients has been stated to be “exceedingly rare” (252). Results with the use of pteroylglutamic acid in the U. S. A. have not been encouraging (253). However, two reports have appeared in England; in one of these (254) the case of a 17-month old boy was described. The patient was emaciated and anemic. He had responded previously to treatment with liver extract, but a second treatment was ineffective. Pteroylglutamic acid, 25 mgm. daily, was administered orally, following which there was immediate clinical improvement and a gain in weight of 13 pounds in 16 days. In the second article (255) two cases of celiac disease (infantile sprue?) were encountered with “typical clinical features” including flat glucose tolerance curves and megaloblastic bone marrow. Both cases responded to daily dosage with 5 mgm. of pteroylglutamic acid with striking clinical improvement and reticulocyte responses of more than 25 per cent.

Glossitis of pellagra. It was reported (256) that the glossitis of pellagra started to respond within two days to the daily oral administration of 10 mgm. of pteroyl glutamic acid. By 5 days the glossitis had disappeared completely in one case and in a second case there was “evidence of a great deal of healing”. The patient had previously shown glossitis which had been successfully treated with niacinamide. These observations may indicate an interesting relationship between niacinamide and pteroylglutamic acid deficiencies in pellagra.

Radiation sickness. Pteroyltriglutamic acid was administered at the rate of 5 mgm. daily for 6 days to 8 patients with leukopenia resulting from local intensive x-ray treatment of carcinoma of the cervix. Elevations of the leukocyte count were noted, but the amounts of pteroyltriglutamic acid available were so limited that the results obtained were not considered to lead to definite conclusions (257). A group of patients with lymphoblastomas received radiation therapy which resulted in an aggravation of anemia and neutropenia. When a crude preparation of pteroylglutamic acid was fed by mouth in addition to the use of radiation therapy in 69 of the patients, it seemed that the deleterious and depressant effects of radiation on the bone marrow were decreased, the frequency of necessary transfusions was lessened, and the period of hospitalization was lessened (258).

Miscellaneous observations. It was noted in India that certain cases of chronic diarrhea responded to treatment with 40 to 60 mgm. of pteroylglutamic acid
daily. Therapy restored the stools to normal or approximately so within two to five days. It was suggested that in long-standing diarrhea a nutritional factor prolonged the production of abnormal stools and that pteroylglutamic acid appeared to correct this defect (259).

The finding that edema occurs in "vitamin-M" deficient monkeys (260) is paralleled by a recent case report of the disappearance of "nutritional edema" in 7 days in a one-year-old child upon treatment with pteroylglutamic acid (261).

### TABLE 5

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>NO. OF CASES</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
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<td>Anemia of premature infants</td>
<td>18</td>
<td>(248)</td>
</tr>
<tr>
<td>Hypochromic macrocytic anemia</td>
<td>5</td>
<td>(248)</td>
</tr>
<tr>
<td>Chronic hypoplastic anemia</td>
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<td>(248)</td>
</tr>
<tr>
<td>Mediterranean anemia</td>
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<td>(248)</td>
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<tr>
<td>Subacute myelogenous anemia</td>
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<td>(248)</td>
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<tr>
<td>Acute lymphatic anemia</td>
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<td>(248)</td>
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<tr>
<td>Sickle cell anemia</td>
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<tr>
<td>Hypoplastic marrow</td>
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<tr>
<td>Monocytic leukemia</td>
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<tr>
<td>Leukopenia in virus influenza</td>
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<td>Macrocytic anemia secondary to cirrhosis</td>
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<td>Anemia associated with portal cirrhosis</td>
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<td>Acquired hemolytic anemia</td>
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<td>Anemia associated with leukemia</td>
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<td>Anemia associated with Weil's disease</td>
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<tr>
<td>Refractory anemia following insecticide exposure*</td>
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<td>Refractory anemia, other causes*</td>
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<tr>
<td>Leukopenia following x-ray treatment* in Hodgkin's disease</td>
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<td>(257)</td>
</tr>
<tr>
<td>Leukopenia following sulfonamide treatment*</td>
<td>1</td>
<td>(257)</td>
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</tbody>
</table>

* Treatment with 5 mgm. pteroyltriglutamic acid daily for 6 days.

Relief of two cases of dermatitis was observed in patients who received supplementation with a crude concentrate of pteroylglutamic acid prepared from liver (262). A synthetic pteroyldiglutamic acid was found to produce remission in two relapsed cases of pernicious anemia and one case of nutritional macrocytic anemia (263). One of the cases received 20 mg. daily by mouth for 10 days.

**Blood dyscrasias not responding to pteroylglutamic acid.** Information regarding these is summarized in table 5.

**Discussion.** An attempt to define two broad groups of megaloblastic anemias
on the basis of recent results with pteroylglutamic acid is made in table 6. No clear-cut differentiation between the two groups is implied, indeed, it seems that sprue embodies certain characteristics of both groups. A metabolic defect in sprue is indicated by its tendency to relapse and by its response to refined liver extract; in one case report (192) an effect of liver extract on pteroylglutamic acid metabolism in sprue is indicated. A dietary deficiency in sprue is implied by the effects of the accompanying diarrhea and frequently by the nutritional history of the patients.

It has been pointed out that some cases of nutritional macrocytic anemia not only fail to respond to the erythrocyte maturation factor but also do not respond to feeding lean beef with or without normal human gastric juice, as a source of the extrinsic factor (18). It has been observed that dietary pteroylglutamic acid deficiency in chicks and rats is not alleviated by lean beef (265).

The metabolic disturbance in addisonian pernicious anemia not only involves pteroylglutamic acid but in some patients also includes neurological disturbances which in certain cases may be controlled by liver extract and not by pteroylglutamic acid.

Further light on the classification of the megaloblastic anemias may result from studies in the utilization of pteroylheptaglutamic acid (p. 86). Another possibility for biochemical investigation may lie in additional studies with thymine, which in large doses has been reported to produce remission of pernicious anemia (p. 84) and sprue (p. 90).

**Excretion.** Only very small amounts of “folic acid” were reported to occur in human urine (266). It was found (50) that the daily urinary excretion of

| TABLE 6 | Differentiation of two groups of megaloblastic anemias |
|-----------------------------|---------------|---------------|
| CHARACTERIZATION | GROUP 1 | GROUP 2 |
| Hemopoietic response to: | | |
| a. Erythrocyte maturation factor of concentrated injectable liver extracts | + | - |
| b. Lean beef + normal human gastric juice | + | - |
| c. Pteroylglutamic acid | + | + |
| Occurrence | May occur on “normal” diet | On deficient diets, usually in tropics |
| Suggested cause | Metabolic defect | Dietary deficiency of pteroylglutamic acid |
| Analogue in experimental animals | Unknown | Pteroylglutamic acid deficiency in monkeys, chickens, rats |
| Examples | Addisonian pernicious anemia | Tropical macrocytic anemia |
Studies of the daily urinary and fecal "folic acid" excretion were measured by assay with *S. fecalis* R (267). Seven young men on a "normal" diet served as experimental subjects. The average daily "folic acid" excretion was about 4 micrograms in the urine and 300 micrograms in the feces. The average daily dietary intake was found to be only 62 micrograms, which seems to be very low, although the diet was not described. In a further communication (268) the effect of supplementation with various vitamins, including 90 micrograms of pteroylglutamic acid daily, was measured in the case of 2 of 7 subjects on a "restricted" diet. Urinary excretion of pteroylglutamic acid was not increased by the supplementation, but the fecal excretion was possibly increased somewhat. Again, the fecal excretion exceeded the intake. The extent to which fecal pteroylglutamic acid is nutritionally available is at present unknown. The occurrence of nutritional macrocytic anemia presumably as a result of dietary pteroylglutamic acid deficiency (4, 21, 22, 23, 24) may indicate that "intestinal synthesis" is ineffective as a source of pteroylglutamic acid under certain conditions, although the production of pteroylglutamic acid deficiency in human subjects on purified diets has not been reported.

It was reported (192) that the daily urinary excretion of pteroylglutamic acid rarely exceeds 5 micrograms and is usually 2 to 4 micrograms on ordinary diets. Oral or parenteral administration of pteroylglutamic acid led within 24 hours to an excretion of 15 to 75 per cent of the amount administered depending on the size of the dose; one individual excreted 16 per cent of one mgm. intramuscular dose, while on a dosage of about 10 mgm. daily either orally or parenterally the excretion usually ranged between 35 and 50 per cent.

Doses of 5 to 16 mgm. of pteroylglutamic acid were fed to 9 normal subjects and 9 hospital patients (269). The normal subjects before receiving pteroylglutamic acid had an average daily urinary excretion of between 2 and 3 micrograms of pteroylglutamic acid. They excreted an average of 28.5 per cent of the administered dose. Most of the excretion took place the second and eighth hours after dosage. The hospital patients excreted much lower percentages of the administered dose. Oral administration of 5 mgm. of sodium pteroylglutamate was found (270) to result in excretion of between 44 and 57 per cent of the administered dose within 6 hours. After 24 hours the excretion fell to basal levels.

**Pharmacology.** Studies of the pharmacology of pteroylglutamic acid indicated that the substance had a low acute and chronic toxicity and showed an almost complete absence of side reactions even when the dose was far above the therapeutic range (271). Mice, rats, guinea pigs, rabbits, cats and dogs were used in the studies. Renal damage was observed in some of the animals following intravenous injection of large doses of sodium pteroylglutamate. The substance did not affect the respiration or the blood sugar and the effects on the blood pressure and the isolated intestine were of a minor order. In chronic experiments, the daily administration of 5 mgm. per kilo intraperitoneally to rabbits and
rats for two months produced no unfavorable reactions. In a similar period, daily intraperitoneal injections of 50 mgm. per kgm. to rabbits and 75 mgm. per kgm. to rats produced some changes in the tubules of the kidney, but no deaths.

The acute toxicity was low. Rats and mice tolerated approximately 200 mgm. per kgm. intravenously, with no evidence of action.

Relation of pteroylglutamic acid to an anemia produced in dogs. An anemia was reported to occur in dogs which received choline by stomach tube or acetyl choline by injection. Acetyl-choline-like activity was detected in trichloracetic acid filtrates of the serum of blood drawn from dogs one to 1 1/2 hours after the oral administration of 200 mgm. of choline chloride. Trichloracetate was not removed from the filtrate before the acetyl choline assay was made. The activity was diminished in the serum of dogs which had previously received liver extract or pteroylglutamic acid. Cholinesterase activity of the serum was increased by incubation with pteroylglutamic acid or liver extract at 37°C. In two normal human subjects, administration of 5 to 7.5 mgm. of pteroylglutamic acid by mouth led to increases of 33 per cent and 16 per cent in their serum cholinesterase activities within 5 hours. It was concluded that liver extract and pteroylglutamic acid acted by increasing the formation of choline esterase (272). Further experiments indicated that the rate of regeneration of the cholinesterase activity of the serum of diisopropylfluorophosphate treated-dogs was increased by the administration of liver extract or pteroylglutamic acid (273). No confirmation of these results has been reported. No anemia-producing effect of choline was observed in various experiments with human subjects (274, 275, 276).

Pteroyltiglutamic acid, pteroylglutamic acid and mammary tumors in mice. A series of studies was made of the effect of intravenous injections of yeast extract on spontaneous breast tumors in mice (277, 278, 279, 280). Spontaneous tumors were studied in the earlier experiments and later (281) tumors were developed in Rockland mice by transplantation with Sarcoma 180. The mice were then used as test animals. They were kept on a normal diet and the effect of four intravenous injections over a period of 48 hours 8 days after the transplantation was judged by comparing sizes and weights of tumors in matched treated and untreated mice. Inositol alone among crystalline factors of the B complex was reported to inhibit the rate of tumor growth over a range of 50 to 250 micrograms daily when administered by intravenous injection, but subcutaneous or oral administration were ineffective (282). Inhibition of tumor growth in this test was reported to be produced by injecting a concentrate of "folic acid" 0.16 to 5.0 micrograms, or by injecting pteroyltiglutamic acid, 0.0063 to 0.40 microgram (305). Studies with spontaneous breast tumors in mice (283) showed that intravenous injections of pteroyltiglutamic acid, 5 micrograms daily, led to complete regressions of spontaneous breast cancers in mice in the case of 43 per cent of a group of 89 animals. No tumors disappeared among the 60 controls. Mice from three strains were used. In contrast, pteroylglutamic acid had no inhibitory effect and it was reported that primary tumors in mice receiving 100-microgram doses of pteroylglutamic acid intravenously grew more rapidly than the tumors of untreated controls (284).
Further experiments with the transplanted tumors were reported (285). Xanthopterin was found to be inhibitory. It was reported that leucopterin antagonized the inhibitory effect of xanthopterin. Studies of the effects of mutually competitive substances on transplanted tumors were reported (286). Inositol was an inhibitor of tumor growth and was neutralized by p-aminobenzoic acid or pyridoxine; desthiobiotin inhibited tumor growth and was neutralized by biotin.

**Microbiological assay methods.** A variety of methods has been proposed (8, 186, 287, 288, 289, 98) for the microbiological assay of pteroylglutamic acid. A number of attempts to improve the basal media have been made by increasing the amounts of vitamins, amino acids and the supplementary factors, and by increasing the amounts of glucose and buffering capacity to permit a higher maximum growth. These media are described in table 7.

### TABLE 7

**Media described for the assay of pteroylglutamic acid**

<table>
<thead>
<tr>
<th>INGREDIENT</th>
<th>AMOUNTS PER 10 ml AND BIBLIOGRAPHIC REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td>Na acetate</td>
<td>60 mgm.</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>60 mgm.</td>
</tr>
<tr>
<td>Na citrate</td>
<td>60 mgm.</td>
</tr>
<tr>
<td>Acid-hydrolyzed casein</td>
<td>50 mgm.</td>
</tr>
<tr>
<td>Glucose</td>
<td>100 mgm.</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.0 µg.</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>1.0 µg.</td>
</tr>
<tr>
<td>Adenine</td>
<td>0.1 µg.</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.1 µg.</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.1 µg.</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.1 µg.</td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.5 µg.</td>
</tr>
<tr>
<td>Norited peptone</td>
<td>Present</td>
</tr>
<tr>
<td>dl-Alanine</td>
<td>Present</td>
</tr>
<tr>
<td>Thiamine</td>
<td>1.0 µg.</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1.0 µg.</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>2.0 µg.</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>1.0 µg.</td>
</tr>
<tr>
<td>Ca pantothenate</td>
<td>5.0 µg.</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.002 mgm.</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>Present</td>
</tr>
<tr>
<td>Salts A†</td>
<td>0.05 ml.</td>
</tr>
<tr>
<td>Salts B‡</td>
<td>0.05 ml.</td>
</tr>
</tbody>
</table>

* For L. casei.
† For S. fecalis R.
‡ K2HPO4, 5 gram; KH2PO4, 5 gram; H2O, 50 ml.
§ MgSO4·7H2O, 10 gram; NaCl, 0.5 gram; FeSO4·7H2O, 0.5 gram; MnSO4·2H2O, 0.337 gram; H2O, 250 ml.
The two organisms which have been used for assay purposes are \textit{S. fecalis} R and \textit{L. casei}. \textit{S. fecalis} has the advantage of giving more reproducible results and of requiring shorter incubation periods than with \textit{L. casei}, but it requires approximately 5 times as much pteroylglutamic acid as \textit{L. casei} to give half maximum growth, thus limiting the assay of very low-potency materials.

Growth with \textit{S. fecalis} R can be measured turbidimetrically after 16 hours or after 40 hours by titrimetric methods. \textit{L. casei} requires an additional growth stimulant to attain maximum growth during the first 16 hours. This growth stimulant, which has been termed "strepogenin" (290) is liberated from casein by tryptic digestion. The acid-hydrolyzed casein usually used as a nitrogen source in microbiological assay media is devoid of strepogenin and thus it is not possible to conduct an assay with \textit{L. casei} in 16 hours for pteroylglutamic acid with such media. A method has been devised (291) to permit a 16-hour assay with \textit{L. casei} by using a tryptic hydrolyzate of casein as a source of amino acids and strepogenin. There have been insufficient published reports on this method, however, to properly assess its value.

In the assay of enzymatically hydrolyzed conjugates \textit{L. casei} will frequently give higher results than those obtained with \textit{S. fecalis}. This is probably due to the fact that \textit{L. casei} will respond to partially hydrolyzed conjugates which are inactive for \textit{S. fecalis} R.

The method of choice would seem to be that of Teply and Elvehjem using either \textit{S. fecalis} or \textit{L. casei}. The medium has a high concentration of buffers and glucose which permits a high maximum growth and contains added quantities of \textit{dl} alanine which has been shown to markedly stimulate growth in the presence of suboptimal amounts of pteroylglutamic acid (292).

The importance of using a pure grade of cotton for plugs is indicated by the observation (293) that nonabsorbent cotton may contain appreciable amounts of pteroylglutamic acid. Pteroylglutamic acid is very labile at pH 7 to direct or indirect sunlight but four hours exposure to artificial light produced only 10 per cent destruction (294).

The need for appropriate enzymatic hydrolysis of conjugates to precede microbiological assay has already been discussed. In the hydrolysis of natural materials two steps probably occur, first, the release of pteroylglutamic acid or its conjugates from the tissue to give a soluble form and second, the hydrolysis of the soluble but inactive conjugates to microbiologically active compounds. It was found that while taka-diastase cannot hydrolyze all the conjugates present (295, 189) it does aid in releasing the factor from tissues (184). Autoclaving at pH 4 or with 2N potassium hydroxide has been found effective (296) in splitting a conjugate obtained from liver. The use of alkaline hydrolysis has the disadvantage that while pteroylglutamic acid is stable to alkali under anaerobic conditions, it is quickly destroyed by alkali in the presence of oxygen (163). It was also found that (163) pteroyltriglutamic acid could be hydrolyzed anaerobically by alkali but that racemization of the pteroylglutamic acid which was released occurred. A comparison was made of a variety of chemical and enzymic methods for the liberation of pteroylglutamic acid (297). Hydrolysis by auto-
claving at pH 4, by autoclaving with 0.1N potassium hydroxide, and by digestion with taka-diastase and with conjugase from chicken pancreas were compared. With certain tissues e.g. chicken kidney, taka-diastase digestion gave higher results than digestion with conjugase from chicken pancreas. However, with other products, such as yeast extract, conjugase treatment gave the highest values. These data show that there is no digestion method which will yield the highest result with all types of products. The authors (127) have found digestion with taka-diastase, followed by hydrolysis with chicken pancreas conjugase to give higher results than treatment of either enzymic preparation alone.

Assay with chicks. The assay of “vitamin B₉” with chicks on a purified diet was described (80). The chicks were placed on the basal diet until they became anemic, following which the supplements were administered. A prophylactic method has been used more recently (186, 298, 299, 300) based on growth and examination of the blood. The chick assay appears to express the total pteroylglutamic acid content of the sample including both the free and conjugated forms (186).

The use of rats and monkeys for assay animals has been described in detail (301).

Distribution of pteroylglutamic acid. A number of studies have appeared which describe the assay of foods and other natural products for pteroylglutamic acid. The interpretation of some of the early results is complicated by several observations. Pteroylglutamic acid exists naturally to a considerable extent in the form of conjugates which are inactive in the microbiological assay until treated with a specific enzyme preparation which liberates pteroylglutamic acid (185). Even when a preparation of the enzyme is used, there is no criterion for the completion of the hydrolysis, and this consideration is brought into sharp focus by the reported existence of inhibitors of the enzyme system in natural foods (240). Attention has been drawn to the growth promoting effect of thymine as a possible complicating factor in the interpretation of the assay (123). The availability of pteroylglutamic acid conjugates in the nutrition of animals appears to be good (37, 68, 70, 92) although questions have been raised as to the utilization of conjugates by certain species (43) and by pernicious anemia patients (235, 236).

The “folic acid” content of a number of natural foods was carried out by assay with S. fecalis R (302). Taka-diastase was used in the preparation of the extracts. This enzyme preparation has been reported not to liberate pteroylglutamic acid completely from its conjugates (36). “Folic acid” showed the greatest losses due to cooking of all the B vitamins studied; cooking losses among meats ranged from 46 per cent in halibut to 95 per cent in pork chops, and in vegetables from 69 per cent in cauliflower to 97 per cent in carrots. The authors postulated that the vitamins may become “bound” in tissues during cooking. Muscle meats ranged from 0.7 to 2.0 micrograms of “folic acid” per gram of fresh tissue, liver 3 to 6 micrograms and vegetables 0.5 to 2 micrograms. The “folic acid” content of samples of various canned foods were assayed for “folic acid” with S. fecalis R and L. casei following incubation with taka-diastase for 24 hours at $37^\circ$ (303). Considerable disparity between S. fecalis and L. casei values was
encountered, for example yellow corn was found to contain 0.017 microgram per gram by S. fecalis R and 0.056 by L. casei. Most of the values obtained with canned foods were quite low, averaging less than 0.1 micrograms per gram, except for canned spinach.

Similar assay methods were applied to a study of meats (304). Muscle meats were found to contain 0.06 to 0.33 microgram per gram of fresh tissue, liver 0.4 to 1.5 micrograms and kidney 0.3 to 0.6 microgram. Cooking resulted in destruction ranging up to 92 per cent of the original content.

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