Technical Papers

The Structure and Synthesis of the Liver *L. casei* Factor

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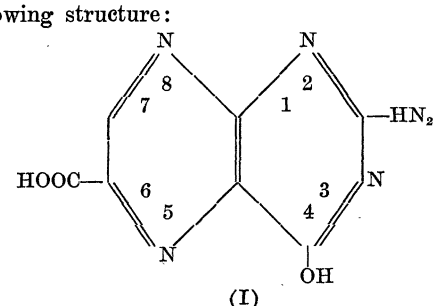
Several compounds have been described which are essential for the growth of *Lactobacillus casei* and which possess hematopoietic activity for animals. One of these compounds was isolated from liver and has been designated the liver *L. casei* factor (*5*). Another form of the compound was isolated from a fermentation product and has been termed the fermentation *L. casei* factor (*3*).

The purpose of this communication is to describe the degradation reactions used to characterize the liver *L. casei* factor and to present two methods of synthesis.

The relationship between the fermentation and the liver *L. casei* factors was shown by anaerobic alkaline hydrolysis which converted the fermentation compound into the dl-liver *L. casei* factor with the simultaneous formation of two moles of a compound which contained alpha-amino acid nitrogen.

On aerobic alkaline hydrolysis of the fermentation *L. casei* factor two fractions appeared to be formed in equimolar amounts, one of which was highly fluorescent while the other fraction gave a positive test for an aromatic amine, using the method of Bratton and Marshall (*2*).

The fluorescent compound was a dibasic acid having pKa values of 3.9 and 7.7. On heating the compound to 300° C., carbon dioxide was evolved, and the resulting product was a monobasic acid having a pKa of 8.0. These data indicate the presence of a monocarboxylic acid. Oxidation of the original compound with chlorine water, followed by hydrolysis with 0.1 N hydrochloric acid gave a positive test for guanidine. The ultraviolet absorption spectrum, fluorescence, empirical formula, and the formation of guanidine indicated the presence of a 2-amino pteridine containing a hydroxy and a carboxylic acid group. This substance was identified by comparison with a synthetic compound as a pteridine having the following structure:



(I)

2-amino-4-hydroxypteridine-6-carboxylic acid

This compound was synthesized by chlorination with phosphorus pentachloride and reduction with hydrogen iodide of 2-amino-4,7-dihydroxypteridine-6-carboxylic acid (*4*). Evidence for the selective chlorination and reduction of the 7-hydroxy group was obtained by decarboxylation of compound (I) and the identification of the product as 2-amino-4-hydroxypteridine by comparison with a compound which was synthesized from 2,4,5-triamino-6-hydroxypyrimidine and glyoxal.

This 2-amino-4-hydroxypteridine-6-carboxylic acid can also be prepared from the corresponding 6-methyl or 6-acetic acid compound, or from the *N*–[(2-amino-4-hydroxy-6-pteridyl)methyl] pyridinium iodide by oxidation with hot alkaline potassium permanganate. These compounds are described elsewhere in this paper.

After acid hydrolysis of the aromatic amine fraction, a compound was isolated and identified as *p*-aminobenzoic acid.

Sulfurous acid cleavage of the fermentation *L. casei* factor gave a pteridine fraction and an aromatic amine. The pteridine fraction reacted rapidly with aldehyde reagents to give insoluble derivatives, which indicated the presence of a carbonyl group. The aldehyde, on standing with dilute alkali in the absence of oxygen, appears to undergo a Cannizzaro-type reaction to yield the previously described 2-amino-4-hydroxypteridine-6-carboxylic acid and another pteridine, which has been identified as 2-amino-4-hydroxy-6-methylpteridine by comparison with an authentic sample prepared by decarboxylation of 2-amino-4-hydroxy-6-pteridineacetic acid. This latter compound was prepared by the condensation of 2,4,5-triamino-6-hydroxypyrimidine and methyl γ,γ-dimethoxyacetoacetate.

Final proof that the methyl group of 2-amino-4-hydroxy-6-methyl-pteridine was in the 6-position was obtained by degradation of the compound by the

method of Weijlard, Tishler, and Erickson (*6*). The resulting product was compared with an authentic sample of 2-amino-5-methylpyrazine and found to be identical.

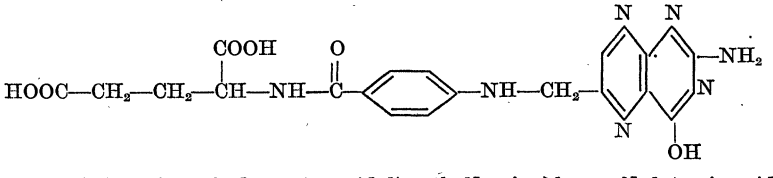
Acid hydrolysis of the amine fraction liberated 75 per cent of the nitrogen as alpha-amino acid nitrogen. From these acid hydrolysates *p*-aminobenzoic acid was isolated and characterized, and by microbiological assay the presence of three moles of glutamic acid was indicated. The presence of the glutamic acid was further substantiated by isolation of pyrrolidonecarboxylic acid from aqueous hydrolysates of the fermentation *L. casei* factor.

No significant amounts of fragments containing one or two carbon atoms could be detected in aerobic alkali or sulfurous-acid-cleaved fractions of the *L. casei* factor.

The parallel liberation of the aromatic amine and the pteridine indicated that the pteridine was attached to the amino group of the *p*-aminobenzoic acid. Since no pteridines containing more than one carbon atom in the side chain could be isolated and since there was no evidence for the existence of one or two carbon atom fragments in the hydrolysis mixtures, a one-carbon atom linkage was indicated. The necessity of oxygen for the alkaline cleavage of the *L. casei* factor suggested the presence of a methylene group. This hypothesis was further strengthened by the fact that the cleavage of *N*-benzyl-*p*-aminobenzoic acid with alkali was accelerated by the presence of oxygen.

The presence of a peptide linkage between *p*-aminobenzoic acid and glutamic acid was obvious from the liberation of alpha-amino acid nitrogen by hydrolysis of the amine fragment.

From a consideration of the above evidence, the structure indicated below was postulated for the liver *L. casei* factor.



N—[4—{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}benzoyl]glutamic acid

The structure of the fermentation *L. casei* factor will be discussed in a subsequent communication.

Proof of the above structure was afforded by two methods of synthesis:

(1) The first method consisted of reacting equal molecular amounts of 2,4,5-triamino-6-hydroxypyrimidine, *p*-aminobenzoyl-*l* (+)-glutamic acid, and 2,3-dibromopropionaldehyde in the presence of an acetate buffer. The resulting crude product contained about 15 per cent by weight of the active compound as determined by microbiological assay. The condensation first gave a dihydro derivative which, during the course of the reaction, was transformed into the aromatic compound.

Purification of the active material was effected by the following procedure: The crude material was dissolved at a concentration equivalent to 400 μg. of the active compound/ml. in 0.2 N sodium hydroxide. Solid barium chloride was added to 0.2 N, and ethanol was added to a concentration of 20 per cent by volume. The precipitate was discarded. The solution was freed of barium, diluted to a concentration equivalent to 100 μg. of the active compound/ml., and adjusted to pH 7.0. The resulting precipitate was discarded and the solution was then extracted three times with 10 volume portions of butanol. The aqueous phase was concentrated to a volume equivalent to a concentration of 400 μg./ml. The solution was adjusted to pH 3.0, chilled to 0°–5° C., and the precipitate was collected. This precipitate was dissolved in 0.1 N sodium hydroxide at a concentration equivalent to 200 μg./ml. and treated with charcoal to remove any residual brown pigments. After filtration, the solution was adjusted to pH 3.0 and the active compound crystallized from hot water. This product had the physical and biological properties described in a previous publication (*1*).

(2) The second method of synthesis was carried out by reacting 2,3-dibromopropionaldehyde with pyridine and then condensing this product with 2,4,5-triamino-6-hydroxypyrimidine and potassium iodide to give *N*-[(2-amino-4-hydroxy-6-pteridyl) methyl] pyridinium iodide. This compound was then reacted with *p*-aminobenzoyl-*l* (+)-glutamic acid and sodium methoxide in ethylene glycol at 140° C. to give a crude product containing about 15 per cent of the biologically active material. The active compound was purified as described above and was identical with the compound prepared by the first procedure.

By the same methods of synthesis, using *p*-aminobenzoic acid instead of *p*-aminobenzoyl-*l* (+)-glutamic acid, a compound was obtained which was active for *Streptococcus faecalis* R but inactive for *L. casei* and the chick.

For the compounds formed from *p*-aminobenzoic

acid and *p*-aminobenzoyl-*l* (+)-glutamic acid, the names pteroic acid and pteroylglutamic acid are suggested.

References

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Famine Edema and the Mechanism of Its Formation[1]

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Edema associated with severe undernutrition was widespread in Europe during and shortly after World War I. No adequate explanation from infectious, cardiac, or renal causes was found. Speculation as to the mechanism of formation of this edema subsided with the demonstration that edema can be provoked in animals by a very low protein diet and with the accumulation of evidence for the general validity of Starling's concept of a filtration balance between hydrostatic and colloid osmotic pressures at the capillary wall. Despite some puzzling facts (*10–12*), by the eve of World War II there was almost universal agreement that "famine edema," as in kidney disease, is produced by a profound depression of the plasma colloid osmotic pressure, this in turn being a result of inadequate dietary protein.

In World War II, as expected, famine edema again appeared on a large scale. Contrary to expectation, however, the theory of simple hypoproteinemic causation was not fully sustained in such observations as were made (*4, 7*), though there was reluctance to abandon entirely this attractive theory (*1, 5, 6, 9*). Data gathered by the Allied Armies indicated that hypoproteinemia was common in famine areas but that it was generally slight in degree and was not closely related to the appearance or severity of edema (*3, 13*).

In this laboratory data were obtained from a controlled experiment with 34 men (volunteers from civilian public service), who subsisted on a European type of famine diet for six months preceded by a control period of three months and followed by three months of controlled "relief" feeding. The diet of whole cereals, potatoes, turnips, etc. provided an average of 49 grams of protein daily and proved to be closely similar to the diets in the less fortunate parts of western Europe in early 1945. These men lost an average of 24.5 per cent of their body weight. Pitting edema appeared within two months in some of the men and eventually in all but a few of the group; even the few apparent exceptions were shown, by special means, to be "waterlogged." At the end of semistarvation the thiocyanate method indicated an average SCN^- space of 34.0 per cent of the total body weight and a relative excess of 7.19 kg. (15.9 lbs.) of extracellular water per man. In proportion to the non-SCN^- space, the extracellular water rose from a "normal" average of 282 grams/kg. of cellular tissue to a "starvation" average of 554 grams/kg.

This development of edema was accompanied by only a small decline in plasma protein concentration, averaging 0.73 grams/100 cc. At the same time the ratio of albumin to globulin decreased to only a trivial extent. Independent analyses by the Tiselius electrophoresis method (veronal buffer) gave an average A/G of 2.00 for the heparinized plasmas of six men who showed marked edema. The same method, when applied to serum, gave A/G values of 1.70 and 1.89 for two of these men. Heparin has a slight influence on the Tiselius pattern which will be reported subsequently.

Clinical edema vanished during three months of refeeding which produced an average recovery of 37 per cent of the lost weight. At the same time the plasma protein concentration returned to normal, but there was a slight fall in A/G; by the Tiselius method A/G averaged 1.82 in the heparinized plasmas of the six men mentioned above. Clearly, the edema was not explicable in terms of hypoproteinemia or subnormal colloid osmotic pressure in the plasma, even with "correction" for protein in the interstitial fluid; edema fluid in this condition is extremely low in protein.

These subjects, like the victims abroad, showed a marked polyuria, profound bradycardia, and no rise in the concentrations of nonprotein nitrogen and chloride in the plasma. The liver was not palpable, and the heart was much diminished in size by X-ray examination. Thiamine deficiency was definitely ruled out by the analysis of food and excreta for thiamine, the reduced size of the heart, and by the absence of signs of polyneuritis. Direct measurements of venous pressure showed that, instead of an increase, there was a markedly subnormal level. At the end of semi-

[1] This work was supported in part under the terms of a contract, recommended by the Committee on Medical Research, between the University of Minnesota and the Office of Scientific Research and Development. Substantial support was provided by the John and Mary R. Markle Foundation, the Sugar Research Foundation, the National Dairy Council, the Service Committees of the Church of the Brethren and of the Society of Friends of the Unitarian Society, and the Mennonites Central Committee. More complete acknowledgments will be made in future full publications on this experiment.