N-D-GLUCOSYLGLYCINE IN PURINE BIOSYNTHESIS

by

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To Jenny,
and to Peter, Pamela
and Christopher.

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N-D-GLUCOSYLGLYCINE IN PURINE BIOSYNTHESIS

I. Introduction

The nutrition of Lactobacillus gayoni 8289[1] was studied by Cheldelin and Riggs (4) who found evidence for a unique requirement of a growth factor found in liver and yeast which could not be replaced by any of the known nutrients or by any of the better described unidentified growth factors. This study was continued by Cheldelin and Nygaard (5,18) who noted the importance of asparagine, nucleotides and exceptionally large quantities of folic acid. The present work began essentially when it was noted that L. gayoni 8289 required heat treatment of its assay medium in order to achieve rapid initiation of growth. The present study has dealt almost exclusively with stimulatory growth factors rather than with essential factors. As can be observed from Figure 1, heavy growth can be achieved with L. gayoni 8289 within 20 to 24 hours at 37° employing filter sterilized medium. However, if the medium is heat sterilized, heavy growth can be obtained in 15 hours. A supplement of crude materials such as yeast extract can reduce the latter time to 14 hours, and raise the top level of growth significantly above the maximum obtained upon the basal medium alone, either heated or unheated.

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[1] For a discussion of the identity of this organism, see Appendix.

The foregoing growth stimulants have been examined and found to be replaceable by glucosylglycine[2], heated glucose[2] and possibly other materials elaborated during heating of the medium. Further study of GG has implicated it as an intermediate in the formation of purines within the cell.

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[2] The following abbreviations will be used throughout this thesis: GG - N-D-glucosylglycine, the ethyl ester will be implied unless otherwise indicated; GA - N-D-glucosyl-D,L-alpha-alanine; FG - N-D-fructosylglycine; HG - heated glucose, see Procedures; carboxamide - 4-amino-5-imidazole carboxamide.

II. Procedures

A. Cultural Methods

1. Lactobacillus gayoni 8289

The method for growing this organism and employing it in nutritional studies followed the procedure of Cheldelin and Nygaard (4,18) with certain modifications. The assay medium and the test solutions were sterilized using Seitz filters and they were transferred aseptically to sterile culture tubes. Usually the assay medium was inoculated in bulk before aliquots were added to their culture tubes. The assays were incubated at 37° for 12 to 17 hours.

Ten ml of assay medium were supplemented as follows: (a) salts A and Tween 80 were increased three-fold, (b) xanthine (20 µg), (c) pyridoxal and pyridoxamine (each, 1 µg), (d) ascorbic acid (8 mg), (e) calcium lactate·$5H_2O$ (6 mg) and (f) asparagine (1 mg). The supplement of acid-hydrolyzed casein was replaced in the more recent experiments by the amino acids recommended by Henderson and Snell (11). A supplement of heated glucose (HG) was prepared by autoclaving 0.8 g glucose in 100 ml one per cent K_2HPO_4. After cooling, 100 ml one per cent KH_2PO_4 were added. One ml aliquots were present in 10 ml of assay medium.

2. General procedure for other lactic acid bacteria

The same general method described in the previous section was employed. The supplement of HG was omitted from the assay medium, and vitamin B_{12} (10 mμg/10 ml) and pantetheine (10 μg/10 ml) were added.

3. Escherichia coli

This organism was employed to study the formation of carboxamide riboside during sulfadiazine bacteriostasis. The method of Greenberg (9) was followed with certain modifications. The culture used was obtained from Dr. W. B. Bollen, Department of Bacteriology, Oregon State College. It was maintained on the medium of Tatum (32) and an inoculum was grown on the medium of Spizizen, Kenney and Hampil (30). A 6 to 8 hour culture was diluted to 0.45 O.D., and one ml was used to inoculate 9 ml of the sulfadiazine medium (9) in which the glucose was replaced by calcium gluconate. This fermentation was incubated 12 hours at 37°.

B. Preparative Methods

1. N-D-glucosylglycine ethyl ester

This compound was prepared by the method of Wolfrom, Schuetz and Cavalieri (34) by condensing D-glucose with glycine ethyl ester in boiling ethanol. The purified compound was white and crystalline, m.p. 106.5-107.5°.[3]

[3] All melting points were uncorrected. A Fisher-John block was employed except where noted.

(literature m.p. 108°). Glucosylglycine (GG) gave negative tests for an amino acid and reducing sugar under conditions whereby positive tests were obtained for glycine and glucose by the ninhydrin test (17) and the aniline acid phthalate test (20), respectively. Positive tests were eventually obtained at higher reaction temperatures. GG did not react with o-dinitrobenzene indicating the absence of an ene-diol structure and fructosylglycine in particular (7,12,15). A solution of GG did not fluoresce under ultra-violet light or absorb in the ultra-violet region. Glucose was the only reducing sugar isolated from a GG hydrolysate by paper chromatography.

2. N-D-glucosylglycine potassium salt

GG ethyl ester was saponified with an equivalent amount of KOH in absolute ethanol. The potassium salt was isolated by centrifugation, washed with absolute ether and dried. Yield: 95 per cent. It possessed "bound" amino and reducing sugar groups, and it did not react with o-dinitrobenzene.

3. N-D-glucosylglycinamide

This compound was prepared by the ammonolysis of the ethyl ester by the method of von Euler and Zeile (33). M.p. 133-135° (literature m.p. 140°).

4. N-D-fructosylglycine ethyl ester

In addition to the preparation of fructosylglycine

(FG) by direct condensation (to be described in the following section), it was also prepared from GG by the Amadori rearrangement by both of the following methods.

a. Method of Hodge and Rist

Procedure B of Hodge and Rist (13), which employed phenylacetone and morpholine as catalysts for the Amadori rearrangement, was followed. The m.p. of the product was 58-70° (literature m.p. 60-75°). This material reacted with o-dinitrobenzene, a more critical test than the m.p.

b. Action of moist ammonia

The method of von Euler and Zeile (33), when conducted in the presence of traces of moisture resulted in the formation of a compound with the characteristics of FG. First crystals, m.p. 53-55°; second crystals, m.p. 57-59°. Both materials reacted with o-dinitrobenzene.

5. Generalized preparative method for N-glycosyl-amino acid esters

The condensation of glucose and glycine ethyl ester in boiling ethanol (34) yielded GG with extensive contamination by brown materials. Although the purification procedure appeared adequate and the final product was apparently GG, the yields were greatly reduced and the entire procedure was time consuming.

When the methyl ester of glycine was employed and the condensation with glucose was conducted in boiling

methanol, no "browning" occurred and the yields were essentially quantitative. The procedure was simple, the condensation was rapid and the isolation merely consisted of evaporation to dryness at low pressures. Furthermore the procedure was applicable to a variety of sugars and amino acids. There were, however, two disadvantages. The methyl esters were hygroscopic and they were unstable unless stored in the deep freeze.

This method is exemplified by the preparation of N-D-glucosyl-D,L-α-alanine methyl ester. 10.3 g D,L-α-alanine methyl ester were condensed with 13.1 g D-glucose by refluxing for 30 minutes in boiling methanol. Two g glucose were recovered by filtration, and some unreacted amine was still evident in the filtrate. The filtrate was evaporated to dryness at low pressures. Yield: 15 g (90 per cent). The residue was dissolved in 5 ml of absolute methanol at room temperature and reprecipitated with 50 ml of dry acetone. The solvents were decanted and the residue was dried at low pressures. The final product was a white, hygroscopic solid, m.p. 61°, which contained "bound" amino and reducing sugar groups.

All of the materials employed in this preparative work were of commercial origin, except the erythrulose which was obtained by the fermentation of erythritol by Acetobacter suboxydans. The method of Charalampous and

Mueller (3) was employed.

C. Analytical Methods

1. Colorimetric

a. Amino Acids. With ninhydrin by the method of Moore and Stein (17); also modified for spraying paper chromatograms.

b. Reducing sugars

i. Aniline acid phthalate method of Partridge (20); also modified for use with solutions. Aldopentoses formed red colors, whereas aldohexoses formed brown colors.

ii. Benzidine method of Horrocks (14). Pentoses reacted in five minutes, hexoses in 10 minutes.

c. Ene-diols. With o-dinitrobenzene by the method of Kuhn and Birkofer (15). A positive test was indicated by the formation of a purple color.

d. Non-acetylatable diazotizable amines. By the method of Ravel, Eakin and Shive (25), by diazotization and coupling with naphthylethylenediamine; also by the modification of Berry et al (1) for spraying paper chromatograms.

2. Paper chromatography

a. Isolation of carboxamide riboside. The method of Greenberg (9) was employed for which 80 per cent aqueous propanol was the solvent.

b. Purification of carboxamide riboside. The method of Greenberg (9) for purifying carboxamide riboside was improved by substituting water-saturated phenol or butanol-acetic acid-water (4:1:5) as the solvents for rechromatographing the isolated material obtained in a. R_f values of carboxamide and its riboside, and other compounds of interest are presented in Table I.

III. Results

A. Effect of Heating Culture Media

It is generally known that heating culture media results in extensive alteration of the reducing sugars, especially when nitrogenous materials and phosphate are also present, or under alkaline conditions. Complex sugars may be broken down into simple sugars, and simple sugars may be epimerized, fragmented and dehydrated. Fragments may be dismutated to yield acids and hydroxylated compounds. The oxidation-reduction potentials may be lowered by the formation of reducing substances as well as by the expulsion of dissolved oxygen. It is also general knowledge that heated media may be stimulatory or inhibitory. A number of instances of stimulation have been reported (6, 19,24,29).

The stimulatory effect of heating L. gayoni culture medium did not appear to be due to a major alteration of the glucose, since in a supplemented medium, growth with glucose was rapid, and the possible effect of acid formation probably was minimized by the efficient buffering of the medium.

In the first experiment, the effect of heating was analyzed in terms of the formation of reducing substances which are known to stimulate lactic acid bacteria. The data presented in Table II show that yeast extract or

heated media stimulated the growth of L. gayoni. On the other hand, ascorbic acid did not stimulate growth on unheated media, although it provided some stimulation in conjunction with the major stimulants. This experiment furnished unambiguous evidence that the formation of reducing materials was not the exclusive result of heating the L. gayoni culture medium.

A series of systematic studies was undertaken next to ascertain the essential reactants in this heat activation effect. First, the nutrients of the medium listed in Table III were omitted in turn from the heated mixture. The individual supplements omitted were heat sterilized separately and restored to the medium after cooling. The subsequent growth responses furnished by these media are listed in Table III, from which it was evident that omission of glucose or amino acids resulted in significant reduction in growth of L. gayoni. A subsequent test showed that when the components of the medium were heated separately or when glucose and amino acids were omitted from the heated mixture, only slight growth stimulation of L. gayoni was apparent (Table IV). Conversely, when glucose and the amino acids were heated together, a considerable amount of growth was observed. This effect was greatly increased by the presence of salts A (a mixture of potassium phosphates, pH 6.8). Heating the complete

medium was not as effective as heating the mixture of glucose, amino acids and salts A, probably because the inert materials interfered in the reaction.

The individual amino acids were heated with glucose and salts A and the reaction products were tested with L. gayoni. It was found that of all the amino acids tested, glycine stimulated growth most significantly, but only when heated with glucose and salts A (Table V). Another significant observation was made involving the effect of heating alpha-alanine with glucose and salts A. In contrast to the effect of glycine, alpha-alanine provided growth inhibition. Subsequent tests have verified this observation and the apparent stimulation observed with some of the other amino acids was shown later to reflect the formation of HG (cf. Figure 3).

When the culture medium was heated, considerable browning occurred and this suggested that some of the reactions involved in forming L. gayoni activity were similar to the "browning" reactions. Some of the primary intermediates in "browning" have been considered to be N-glucosylamino acids. The corresponding glycine derivative was then prepared and tested for L. gayoni activity. Graded levels of GG, as the ethyl ester, were compared with graded levels of yeast extract for growth stimulation of L. gayoni. In addition, GG was autoclaved in phosphate

buffer (pH 9.5) and then tested at comparable levels. The results have been presented in Figure 2. At concentrations below 4 mg/10 ml, unheated GG stimulated growth of L. gayoni. Above that concentration, GG was inhibitory. The shape of the GG dosage-response curve was sigmoidal and did not correspond to the control curve of yeast extract. When GG was heated with alkaline phosphate buffer its dosage-response curve was considerably altered. Generally, the potency of GG was reduced. However, the shape of its curve now corresponded to that of yeast extract and there was no evidence of toxicity at high concentrations of supplement. It was concluded that heating glucose and glycine formed GG first, and then converted it into some heat stable compound which may represent the functioning form of the L. gayoni stimulatory factor.

That portion of the GG curve (Figure 2) which indicated growth stimulation, was, as noted, sigmoidal. Such a curve is frequently associated with nutritionally deficient media when several limiting growth stimulants are being increased simultaneously. It was considered next that some other growth factor might be formed by heating and to this end, glucose was autoclaved with the alkaline phosphate buffer and used to supplement the unheated GG. It was observed from the data presented in Figure 3 that HG was without significant effect when added

to the assay medium, but when HG was added with GG, it enhanced the GG response in excess of the amount expected from the sum of the individual stimuli. Significantly, in the presence of HG, the GG dosage-response curve corresponded to that of yeast extract. It was concluded that a second growth factor was being formed by heating the culture medium, and that this factor was derived from glucose under alkaline conditions.

A survey of other lactic acid bacteria was undertaken to investigate their requirements for GG. In addition, the inhibitory effect of "glucosylalanine", as represented by an autoclaved mixture of glucose, salts A and alpha-alanine, was also tested with these organisms. Such a study revealed that only a few of the lactic acid bacteria tested (about one-third) responded to GG and only two had appreciable requirements for GG. Significantly those organisms that responded to GG were also inhibited by "glucosylalanine" (Table VI). It was concluded that GG was an essential nutrilite for only two organisms, but it was a metabolite for several more.

In this preliminary survey of lactic acid bacteria, it was soon noted that more organisms were stimulated by heated culture media than were stimulated by GG or by HG. An analysis of this phenomenon led to the following generalizations: Type A, organisms which responded to

heated media; Type B, organisms which responded to heated media or yeast extract; Type C, organisms which responded to GG, heated media or yeast extract; Type D, organisms which responded to HG, heated media or yeast extract. Evidence for this contention is presented in Table VII. All of the organisms tested, which required heated media, could be classified in one of these categories.

B. N-glycosylamino acids

The preliminary studies suggested that the L. gayoni growth factor formed by heating culture media, possessed a certain amount of specificity, inasmuch as glycine was the only amino acid which furnished activity, and alpha-alanine was inhibitory. The activity furnished by GG tended to confirm this hypothesis. It was of considerable interest to prepare an amino acid analog of GG and a logical compound to be attempted was glucosylalanine (GA). Two preparations were made and each was tested for its L. gayoni activity. In the first assay (Table VIII), GA inhibited the action of GG, while in the second assay (Table IX), GA appeared to be inert. The first preparation had been stored at room temperature in a desiccator over calcium chloride and within a short time, had turned light yellow. The second preparation was stored in the deep freeze and was tested shortly thereafter. In addition, the culture medium employed in the first assay was not completely

adequate as is indicated by the relatively light growth obtained. It was concluded that GA was inert, but some product of GA was antagonistic toward the action of GG. This interpretation was consistent with the expressed view that GG *per se* was not the actual *L. gayoni* activity, but some heat stable derivative of it.

In view of the innate reactivity of the glucosyl fragment of GG (although no decomposition products were evident in the crystalline material), the glycosyl analogs of GG were compounds of interest. Many of the reactions involving epimerization, fragmentation and dehydration of sugars occur more readily when the sugars are condensed with an amino acid, and the alterated products may retain the amino acid. In this connection, Gottschalk and Partridge (8) observed that N-glucosyllysine was converted to N-5-hydroxymethylfurfuryllysine through dehydration involving N-fructosyllysine as an intermediate. Undoubtedly, fragmentation reactions too may retain the amino acid. In a series of experiments, a variety of alpha-hydroxy carbonyl and dicarbonyl compounds were reacted with glycine methyl ester and these condensation products were tested for *L. gayoni* activity. In the first series, a group of hexoses were compared. Three of them (glucose, fructose and mannose) were epimers and were presumably

interconvertible by means of the Amadori rearrangement. The fourth, galactose, was planned to be a control for the first group, although theoretically, it might be derived from them by the conversion of the 1,2 ene-diol into a 2,3 ene-diol. The ethyl and methyl esters of GG were also compared. From the data presented in Table X, it was clearly evident that all compounds were active for L. gayoni. All of the methyl esters were nearly equally active, but they were only about 10 per cent as active as GG ethyl ester. In the next series, alpha-hydroxy carbonyl compounds of varying chain length from two to six carbons, were condensed with glycine methyl ester and tested for L. gayoni activity. All the preparations, except those involving glycolaldehyde and glyceraldehyde, formed uncolored products. The latter two compounds "browned" during the reactions. All of these compounds were active for L. gayoni (Table XI). Two of them, the glycolaldehyde and glyceraldehyde analogs were somewhat more active than GG methyl ester, although at higher concentrations, inhibitions were observed. In the last sequence, a group of dicarbonyls were reacted with glycine methyl ester. These products all browned, but they possessed L. gayoni activity (Table XII) which was somewhat in excess of that obtained with GG methyl ester. The toxic substances were shown in later experiments to be due

to the unreacted dicarbonyls.

This series of experiments clearly indicated that the second stage in the formation of L. gayoni activity consists of fragmentation of the carbohydrate portion. Although the final product is still unknown, possibly some type of cyclization is involved. The minimum structural requirement for the glycosyl moiety may be a two carbon alpha-hydroxy carbonyl or dicarbonyl. Such a compound, as glyoxal, could serve as a precursor to the imidazole ring.

C. Glucosylglycine and Purine Biosynthesis

The previous studies have shown the inflexible requirement for glycine and the relatively non-specific contribution of the glycosyl fragment for L. gayoni activity. This suggested a study of GG in known biochemical reactions involving glycine, notably in purine biosynthesis.

When E. coli is grown in simple glucose-salts medium in the presence of a sulfonamide, an amine accumulates (31), which has been identified as 4-amino-5-imidazole carboxamide (28), a precursor of the purines (16). The functional form in which carboxamide exists in the fermentation medium is the ribotide (10). During isolation, the ribotide is hydrolyzed and the carboxamide (31) or the riboside (9) are isolated depending on the severity of the procedure. Best yields of carboxamide are obtained when glucose (31) and glycine (25) are present in the

medium, and these facts prompted an investigation of the effectiveness of GG in replacing equivalent amounts of glucose and glycine. These results are presented in Table XIII. They show that GG produced greater quantities of carboxamide riboside than did the equivalent amounts of glucose and glycine. The greater efficiency of GG utilization suggested a direct conversion of GG into carboxamide riboside.

The postulate of direct conversion of GG into the imidazole ring of purines implies a specific contribution of the glycosyl fragment to this synthesis which was at odds with the results of L. gayoni. In the next experiment, GG, as the potassium salt, ethyl ester or amide, was compared with FG for the stimulation of carboxamide formation. The results shown in Table XIV revealed a surprisingly high degree of structural specificity involved in carboxamide formation. GG as the ethyl ester and the potassium salt, but not as the amide, stimulated carboxamide formation more extensively than the equivalent amounts of glucose and glycine. Furthermore, FG, like GG amide, did not stimulate the synthesis of carboxamide appreciably above the basal, and their "bound" glycine apparently was not available for synthesis. That GG amide was not active might have been anticipated since Ravel, Eakin and Shive (25) had reported glycinamide to be inactive in carboxamide

synthesis. On the other hand, these authors also reported N-formylglycine to be inactive, and in extension, all N-glycosylglycines might also have been expected to be inactive.

In the two previous studies, GG was added only to replace the glycine supplement, and not to replace the carbon and energy source. In the next experiment, the glucose supplement was replaced entirely by FG or by GG, as the ethyl ester, potassium salt or amide. This was a simple growth type of experiment and no sulfadiazine was present in the medium. The results, shown in Figure 4, are of considerable interest. While E. coli readily metabolizes glucose as the carbon and energy source, the utilization of the glycosylglycines was delayed. E. coli began to use the amide and the ethyl ester of GG about two hours after it had begun to use glucose, but in sharp contrast to the utilization of glucose, appreciably more growth was supported by the amide and the ester. These results were not explainable in terms of the added glycine, since glycine did not facilitate the utilization of glucose, but actually reduced the amount of growth obtainable from that source. Neither was it likely that the glycosylglycines were being hydrolyzed to free glucose before being metabolised, since GG as the potassium salt which was presumably as susceptible to hydrolysis as the ester or the amide, was

not utilized by E. coli until well after 10 hours. Moreover, FG as the ethyl ester did not support any growth within 23 hours, although free fructose would be an adequate substrate for E. coli.

D. E. coli filtrate and L. gayoni stimulation

The role of GG in purine biosynthesis with E. coli suggested that purines of their precursors might stimulate the growth of L. gayoni. However, purines were routinely added to the L. gayoni medium, as well as a supplement of yeast nucleotides. Under these circumstances, it is conceivable that portions of the metabolic pathways in the two organisms overlap and that certain intermediates for one might stimulate the other, although their final products may not be mutually functional. Specifically, the possibility was implied that carboxamide or its riboside could stimulate the growth of L. gayoni. The riboside was isolated from the culture filtrate of sulfadiazine inhibited E. coli by paper chromatography. That fraction containing the riboside, as well as the remaining portions of the chromatogram were tested for stimulation of L. gayoni. Neither carboxamide nor its riboside stimulated L. gayoni, but L. gayoni activity was found in the portion of the chromatogram corresponding to R_f 0-0.35. In contrast to GG, this fraction became active only after it had been autoclaved with the L. gayoni medium. The test was

repeated with another chromatogram and those portions corresponding to R_f 0-0.1, R_f 0.1-0.2, R_f 0.2-0.3 and R_f 0.3-0.4 were tested. Again, the riboside contained in the fraction R_f 0.3-0.4, was found to be inactive (Table XV). L. gayoni activity was located in the fraction R_f 0-0.1 where it remained obscured until it was heated with the medium.

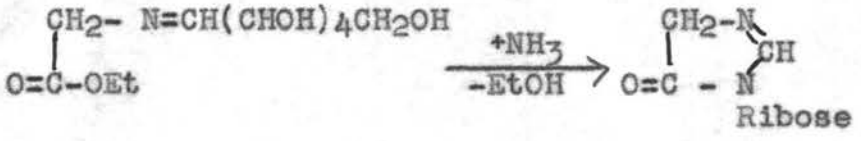
IV. Discussion

The structural requirements for the various biological activities of a series of N-glycosylamino acids have been summarized in Table XVI. From the data available, the requirements for the glycosyl fragment appeared to be more critical for *E. coli* than for *L. gayoni*, since only GG, but not FG, was active, whereas both were active for *L. gayoni*. The study of the amino acid contribution was not as complete as the corresponding study of the glycosyl fragments, although it would be expected that carboxamide formation, like *L. gayoni* activity formation, would be glycine dependent. In contrast to their *L. gayoni* activities, the GG amide was inactive for carboxamide production and the potassium salt of GG was only slowly utilized as an energy source by *E. coli*. These specificities may be suggestive of the mechanism of each reaction, as will be discussed later.

Other glucosylamino acids should be tested with *E. coli* for carboxamide production and for energy utilization. If other glucosylamino acids are inactive for carboxamide formation, then the pathway from GG to purines may be as direct as is suggested by the data of Table XII. On the other hand, if other glucosylamino acids are active for carboxamide formation and also for energy utilization, then it might be considered that GG was being transformed

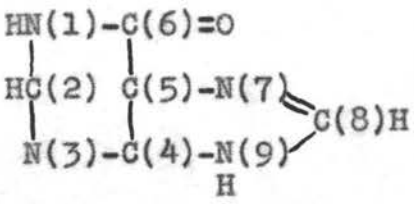
into glucose-1-phosphate by the exchange of the glycine for inorganic phosphate, somewhat in the manner whereby polysaccharides exchange a glycosyl group for inorganic phosphate. The glucose-1-phosphate thus formed from GG would be available for energy utilization by either glycolysis or the hexosemonophosphate shunt. The role of GG then might be as a by-pass mechanism for the initial phosphorylation of glucose, and the stimulation of carboxamide formation might only be a reflection of a higher metabolic turnover of the substrate.

The latter idea, although attractive as the possible mechanism of utilization of GG as an energy source, is not as satisfying as the concept of the direct conversion of GG into purine precursors. It will be noted that GG plus ammonia contain all of the elements needed for the formation of imidazolone riboside, as shown below:



Such a mechanism is predicated on the hypothesis that glucose can break down into a five carbon unit ("ribose") and a one carbon unit ("formate"). It is generally known that the intact glycine molecule is incorporated into the imidazole ring of the purines to furnish the atoms at

positions 4, 5, and 7, as indicated below:



The nitrogen atoms at positions 1, 3, and 9 are derived from metabolic ammonia, and the carbon atom at position 6 is obtained from metabolic carbon dioxide. Although the carbon atoms at positions 2 and 8 are nominally derived from formate, the mechanisms for their insertion into the purine ring do not appear to be identical. The formate going into position 2 is closely associated with the function of a folinic acid coenzyme, whereas the formate going into position 8 is apparently not as closely related. This is evident from the fact that carboxamide, which is a "purine" lacking a carbon atom at position 2, accumulates during sulfonamide bacteriostasis (i.e., during inhibition of folinic acid function when formate can not be inserted to form the purine ring). Furthermore, labeled exogenous formate in the presence of the folinic acid coenzyme, rapidly equilibrates with the carbon atom at position 2 of the purines, but not with the carbon atom at position 8. If the function of folinic acid coenzyme is limited to transformylation reactions, then the process

involved in the insertion of formate at position 2 would be transformylation, whereas another process would seem to be functioning for the insertion of formate into position 8. It is possible that "formate" may be formed directly at the glycine amino group, possibly from GG with no transformylation being necessary. To account for the insertion of formate into position 8 and the attachment of ribose to position 9, it is worth speculating that glucosyl moiety of GG may fragment to form these one and five carbon units which are put in place during the formation of the imidazole ring.

In this connection, Goldthwait and Peabody (6a) have reported the isolation of N-formylglycinamide ribotide as an intermediate in carboxamide synthesis. Presumably, this aliphatic ribotide cyclizes to form the imidazolone ribotide which is then transformed into purine nucleotides via carboxamide ribotide.

It is interesting to note, that neither glycine nor GG can be utilized for carboxamide formation in the form of their amides. This suggests that amide formation is a key intermediate and it may involve an amide linkage with the enzyme. Another interesting speculation deals with the possibility that GG is actually converted to glucose-1-phosphate for energy utilization. Then the slow utilization of the potassium salt by E. coli may

reflect the repulsion of the approaching negatively charged phosphate ion by the negatively charged glycinate ion already attached to the glucose.

In view of the biological activity of GG for both E. coli and L. gayoni, it is speculated that L. gayoni may be able to use a carboxamide precursor for growth, such as imidazolone or its derivatives. This compound could be converted into 4-aminoimidazole by transamination. In this connection Radin and Barker (23a) have shown that the metabolism of purines goes by a route which does not include carboxamide as an intermediate, but possibly instead through 4-aminoimidazole.

V. Conclusions

Many lactic acid bacteria require the stimulus of compounds which are formed in the culture medium by the action of heat on reducing sugars and amino acids. One of these compounds has been identified as N-D-glucosylglycine. Thus glucosylglycine shortens the time needed by *Lactobacillus gayoni* to attain maximum growth on an unheated culture medium. In addition, glucosylglycine increases the formation of 4-amino-5-imidazole carboxamide (an intermediate in purine biosynthesis) by sulfonamide-inhibited cultures of *Escherichia coli*, and for that reason, glucosylglycine may be a direct precursor to the purines. The relation of the *L. gayoni* factor to purines has not as yet been elucidated, but their biosynthetic mechanisms appear to be interrelated.

VI. Figures and Tables

FIGURE 1

L. gayoni growth curves on heated and unheated basal and on unheated basal supplemented with glucosylglycine and yeast extract.

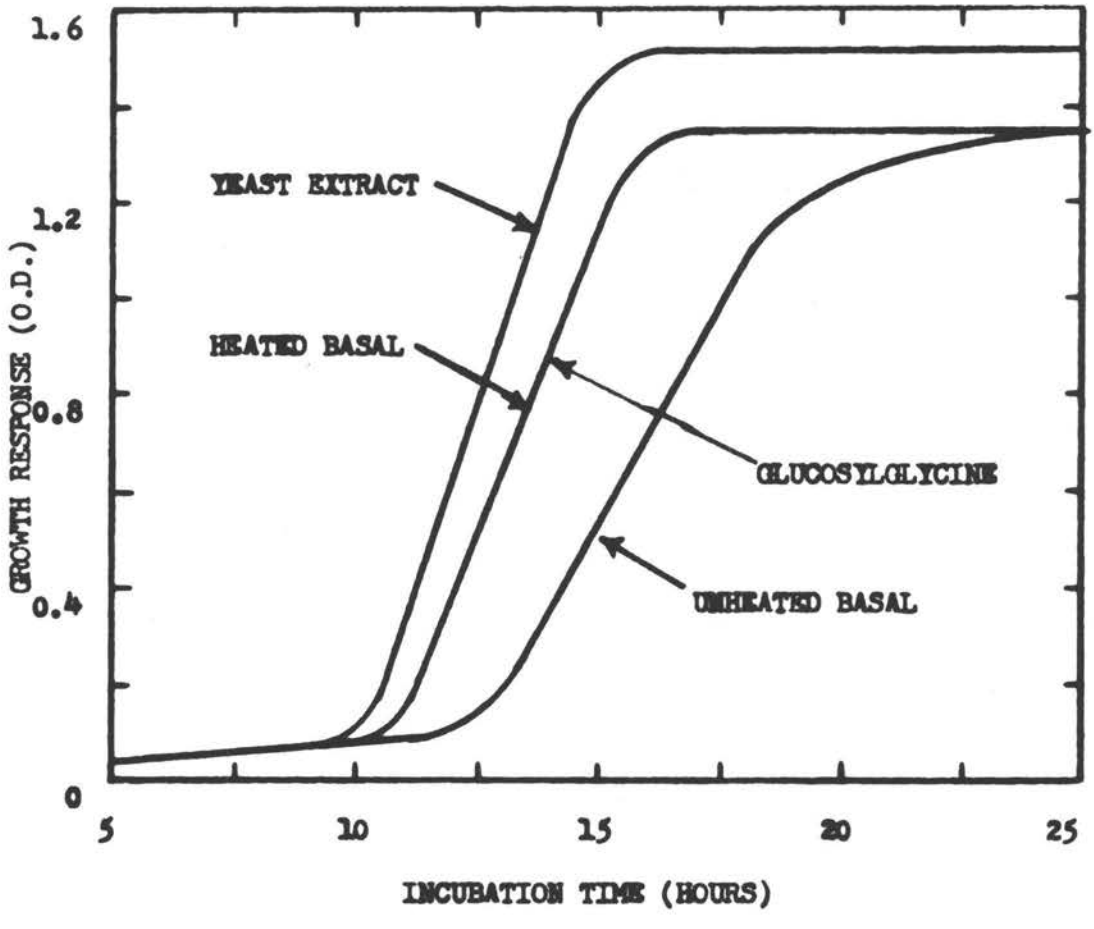


FIGURE 2

L. gayoni response to graded levels of GG, unheated and heated with alkaline phosphate buffer, and yeast extract.

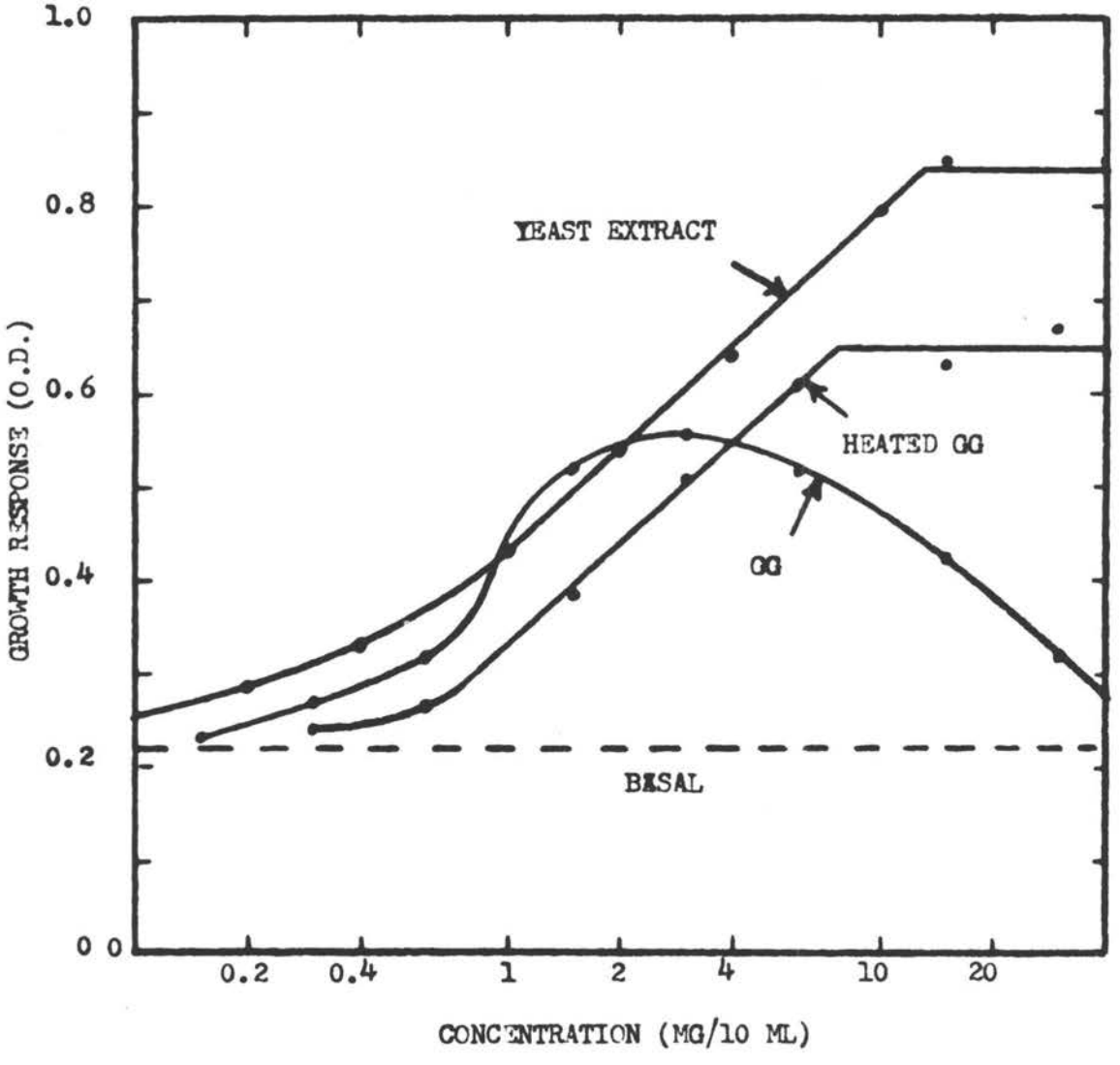


FIGURE 3

L. gayoni response to graded levels of yeast extract and combinations of GG and HG.

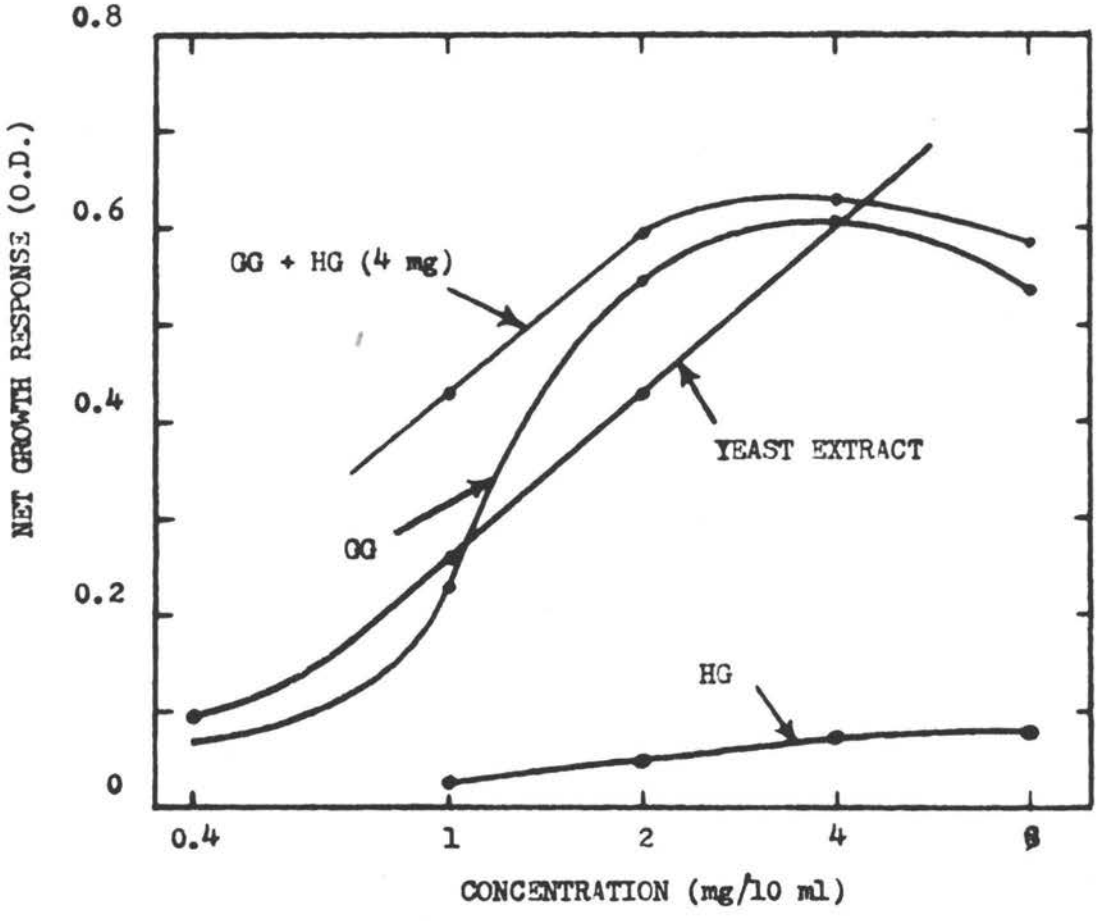


FIGURE 4

The utilisation of glucose and glycine, FG and various forms of GG as energy substrates by E. coli.

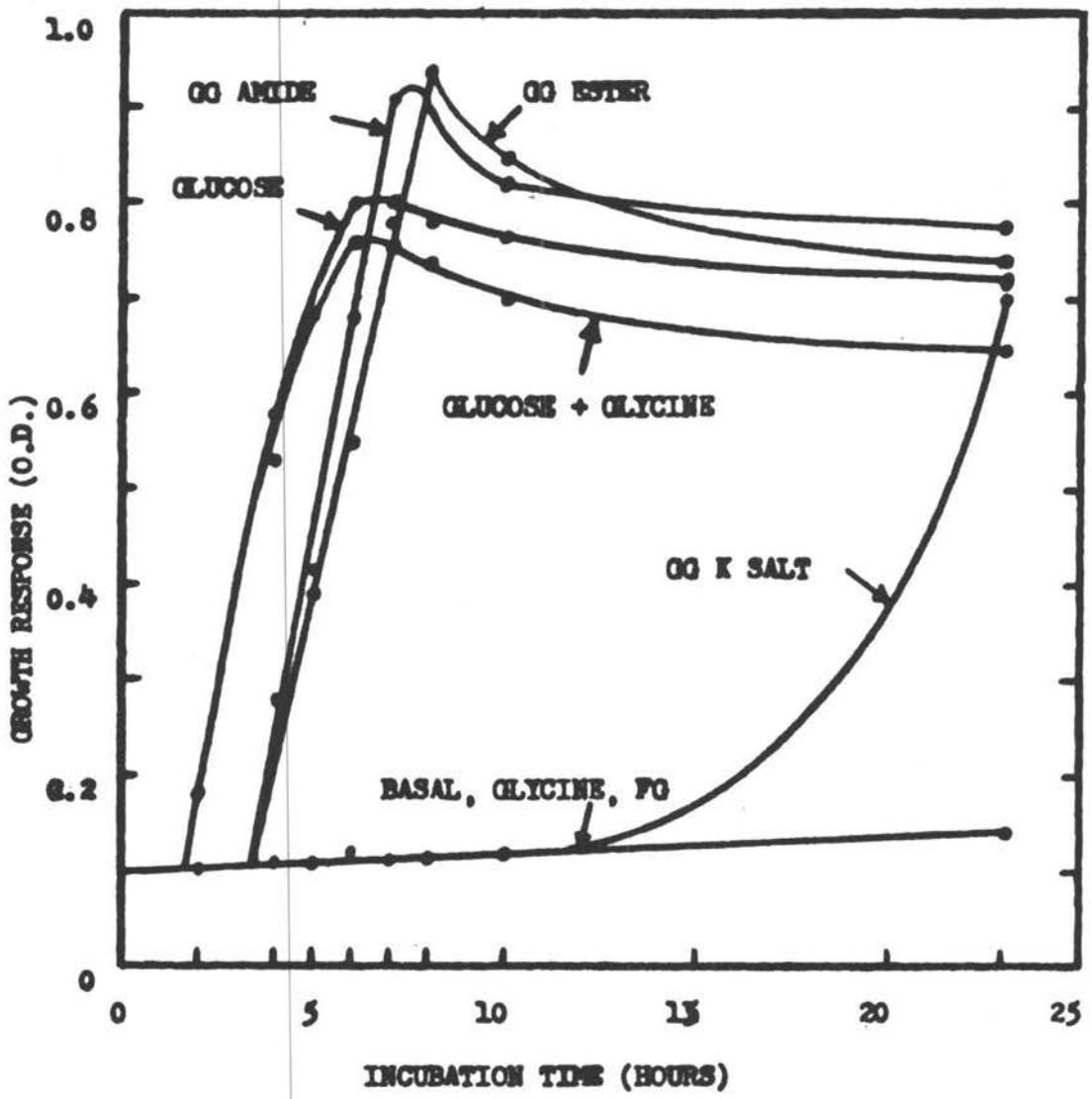


Table I. Paper partition chromatography of carboxamide and its riboside, and other compounds of interest in the culture filtrates of sulfonamide-inhibited E. coli. (The numbers listed are R_f-values.)

Compounds	Solvent Systems		
	propanol-water (4:1)	water saturated phenol	butanol-acetic acid-water (4:1:5)
sulfadiazine	----	0.98	0.85
carboxamide	0.50	0.86	0.48
carboxamide riboside	0.40	0.78	0.39
ribose	0.48	0.64	0.34
glucose	0.35	0.43	0.25
GG-K salt	0.12	----	----
GG-amide	0.25	----	----
GG-ethyl ester	0.63	----	----
glycine	0.15	----	----

Table II. L. gayoni response to yeast extract and heated medium as influenced by ascorbic acid. (The numbers listed represent the turbidity of the assay tubes in optical density (O.D.) units.)

Supplements	Filter-sterilized Medium		Heat-sterilized Medium	
	None	Ascorbic Acid (4 mg)	None	Ascorbic Acid (4 mg)
None	0.19	0.23	0.73	0.89
Yeast extract (50 mg)	0.79	1.02	1.17	1.24

Table III. The effect of omission of the individual components of the medium during heating upon the subsequent L. gayoni growth.

Components Omitted During Heating*	Turbidity of the Assay Tubes (O.D.)
None (all heated together)	0.60
Glucose	0.46
Amino acids	0.53
Acetate	0.65
Vitamins	0.76
Purines, pyrimidines, ammonia-hydrolyzed yeast nucleic acid	0.70
Tween 80	0.68
Salts A	0.65
Salts B	0.79
Ascorbic acid	0.63

* Components listed were sterilized separately and added to the remainder of the medium after cooling.

Table IV. The effect of heating various combinations of medium components upon the subsequent L. gayoni growth.

Components Heated Together*	Turbidity of the Assay Tubes (O.D.)
None (all heated separately)	0.155
All heated together, except glucose and amino acids	0.20
Glucose and amino acids	0.465
Glucose, amino acids and salts A	0.92
All heated together	0.67

* Components not listed were sterilized separately and added to the remainder of the medium after cooling.

Table V. The effect of heating individual amino acids with glucose and salts A upon the subsequent L. gayoni growth. The components not listed were sterilized separately and added to the remainder of the medium after cooling.

Amino Acid Heated With Glucose, Salts A*	O.D.#	Amino Acid Heated With Glucose, Salts A*	O.D.#
None	0.27	D,L-methionine	0.22
Casein hydrolysate	0.51	L-lysine·HCl	0.41
Glycine	0.64	L-histidine·HCl·H_2O	0.39
D,L-alpha-alanine	0.12	L-arginine	0.39
Beta-alanine	0.42	D,L-aspartic acid	0.36
D,L-valine	0.41	L-tyrosine	0.35
D,L-norvaline	0.41	L-glutamic acid	0.42
D,L-leucine	0.34	D,L-phenylalanine	0.30
D,L-isoleucine	0.39	D,L-tryptophane	0.38
D,L-serine	0.25	L-proline	0.29
D,L-threonine	0.36	L-hydroxyproline	0.25
L-cystine	0.18		

* 4 mg amino acid, 100 mg glucose, 0.15 ml salts A; total volume: 0.4 ml.

Turbidity of assay tubes.

Table VI. The stimulation of a group of lactic acid bacteria by GG and their inhibition by "glucosylalanine". (The numbers listed represent the turbidity of the assay tubes in O.D.)

Organism	Experiment A		Experiment B	
	Basal Medium	Glucosyl-glycine	Basal Medium	"Glucosyl-alanine"
L. gayoni 8289	0.165	0.92	0.20	0.135
L. gayoni 49	1.02	1.13	0.63	0.06
L. acidophilus OSC	0.33	0.685	0.26	0.135
L. acidophilus Texas	0.375	0.51	0.325	0.265
L. helveticus	0.185	0.325	0.27	0.005
L. pentoaceticus	0.575	0.69	0.355	0.095
L. plantarum	0.79	0.86	0.505	0.20
L. arabinosus	0.91	0.96	0.375	0.22

Table VII. Typical responses of lactic acid bacteria to products formed by heating culture media. (The numbers listed represent the turbidity of the assay tubes corrected for basal growth; net O.D.)

Organism	Glucosyl-glycine* (4 mg)	Yeast Extract* (4 mg)	Heated Basal	Heated Glucose* (4 mg)
Type A				
L. gayoni F-20	-0.01	0.01	0.81	0.06
L. fermenti 9338	-0.01	0.01	0.65	0.05
Type B				
L. casei	-0.01	0.46	0.46	0.05
Type C				
L. gayoni 8289	0.52	0.49	0.62	0.15
L. acidophilus OSC	0.375	0.44	0.53	0.11
Type D				
S. zymogenes 10100	0.01	0.50	0.50	0.25

* Added to unheated basal medium.

Table VIII. The effect of glucosylalanine (GA; preparation 1) on the L. gayoni response to GG.

Supplements	Turbidity of the Assay Tubes (O.D.)	
	Assay A	Assay B
None	0.055	0.11
GG (4 mg)	0.145	0.195
GA (4 mg)	0.04	0.11
GG (4 mg) + GA (4 mg)	0.045	0.12

Table IX. The effect of glucosylalanine (GA; preparation 2) on the L. gayoni response to GG. (The numbers listed represent the turbidity of the assay tubes in O.D.)

GA (mg)	GG (mg)			
	0	0.4	4	40
0	0.09	0.11	0.72	0.60
0.04	0.07	0.115	0.715	0.59
0.4	0.09	0.13	0.74	0.615
4	0.08	0.12	0.72	0.605
40	0.12	0.14	0.63	0.555

Table X. L. gayoni responses to derivatives of glycine methyl ester and hexoses. (Numbers represent turbidity of the assay tubes in O.D.)

Test Materials	Supplement Level (mg)		
	0.4	4.0	40
Glucosylglycine, ethyl ester	0.28	0.74	0.50
Glucosylglycine, methyl ester	0.24	0.37	0.74
Fructosylglycine, methyl ester	0.25	0.32	0.71
Mannosylglycine, methyl ester	0.22	0.30	0.77
Galactosylglycine, methyl ester	0.24	0.37	0.73
No supplement (basal)	0.24		

Table XI. L. gayoni responses to derivatives of glycine methyl ester and alpha-hydroxy carbonyl compounds of varying lengths. (Numbers represent turbidity of the assay tubes in O.D.)

alpha-Hydroxy carbonyls	Supplement Level (mg)		
	0.4	4.0	40
Glycolaldehyde	0.40	0.60	0.65
Glyceraldehyde	0.42	0.62	0.04
Dihydroxyacetone	0.37	0.45	0.85
Erythrulose	0.37	0.40	0.71
Ribose	0.36	0.41	0.81
Arabinose	0.37	0.49	0.99
Xylose	0.37	0.44	0.90
Glucose	0.39	0.55	1.00

Table XII. L. gayoni responses to derivatives of glycine methyl ester and alpha,beta-dicarbonyl compounds of varying lengths. (Numbers represent turbidity of the assay tubes in O.D.)

alpha,beta-dicarbonyls	Supplement Level (mg)		
	0.4	4.0	40
Glyoxal	0.42	0.27	no growth
Methylglyoxal	0.46	0.31	no growth
Diacetyl	0.42	0.78	0.19
Glucose (reference compound)	0.39	0.57	1.13

Table XIII. The influence of glucose, glycine and GG on the formation of carboxamide riboside by sulfadiazine-inhibited E. coli.

Supplement	Concentration (μ mols/10 ml)	Carboxamide Riboside Formed		Calculated Conversion of Supplements to Carboxamide Riboside (per cent)
		Total (μ mols)	Net (μ mols)	
None	----	0.22	----	----
Glucose	4.0	0.25	0.03	0.75
Glycine	4.0	0.52	0.30	7.5
Glucose + Glycine	each 4.0	0.54	0.32	8.0
GG	3.8	0.68	0.46	12.1

Table XIV. The influence of glucose and glycine, fructosylglycine (FG) and various forms of GG on carboxamide formation by sulfadiazine-inhibited E. coli.

Supplements	Concentration (μ mols/10 ml)	Carboxamide Riboside Formed (μ mols)
None	---	0.27
GG ethyl ester	3.8	0.54
GG K salt	3.6	0.51
GG amide	4.2	0.29
FG ethyl ester	3.8	0.29
Glucose + glycine	each 4.0	0.44

Table XV. The isolation of L. gayoni activity from sulfadiazine-inhibited E. coli filtrate by means of paper chromatography.

Supplements	Filter-sterilized Medium	Autoclaved With Medium
None	0.58	0.80
GG (4 mg)	0.87	0.98
R_f 0.0-0.1 (=8 ml)*	0.63	1.085
R_f 0.1-0.2 (=8 ml)*	0.68	0.86
R_f 0.2-0.3 (=8 ml)*	0.64	0.82
R_f 0.3-0.4 (=8 ml)*#	0.64	0.83

* Samples equivalent to 8 ml of the culture filtrate were taken for assay.

Containing 30 µg carboxamide riboside.

Table XVI. Relation of structure and activity of N-glycosylamino acids for carboxamide production, energy substrate for E. coli and L. gayoni growth stimulation.

Structural Form	Structural Requirements for Biological Activity		
	Carboxamide formation by E. coli	Energy substrate for E. coli	L. gayoni growth
N-glucosylamino acid			
glycine	active	active	active
alanine	no test	no test	inert-inhibitory*
others	no test	no test	no test
N-glycosylglycine			
glucose	active	active	active
fructose	not active	not active	active
others	no test	no test	active
N-glucosylglycine			
ethyl ester	active	active	active#
methyl ester	active	no test	active#
potassium salt	active	slowly active	active#
amide	not active	active	active#

* Decomposition products may be antagonistic to GG.

\# Relative activities: ethyl ester = amide > methyl ester > potassium salt.

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VIII. Appendix

VIII. Appendix

Lactobacillus gayoni was isolated and described by Pederson in 1929 (21,22) as a new species, although nine years later he reclassified this organism as L. fermenti (23). There are serious questions today concerning the validity of the present classification of this organism. Undoubtedly certain strains originally labeled "L. gayoni" are very similar to L. fermenti and their reclassification is probably justified. However, there are also cultures originally labeled "L. gayoni" which, on the basis of several criteria, are distinct from cultures of L. fermenti. These cultures should be restudied and their status legitimatized.

L. gayoni 8289 was obtained from the American Type Culture Collection, Washington, D. C. This strain is listed as L. fermenti and it was obtained indirectly from Pederson. There are points of similarity between L. gayoni 8289 and L. fermenti 9338 because both organisms utilize carbohydrates in a similar manner. According to Pederson (21,22,23) and to the present work, both organisms utilize glucose, galactose, lactose, sucrose, but not xylose. There is a minority report however stating that L. gayoni 8289 can utilize xylose (2). These workers indicated that nearly twice as much lactic acid was produced from xylose as from equivalent amounts of glucose or

galactose, and they observed no acid production at all from lactose and sucrose.

On the other hand, L. gayoni 8289 has several characteristics which distinguish it from L. fermenti 9338 and L. gayoni F-20 obtained from Pederson. First, L. fermenti 9338 and L. gayoni F-20 can grow rapidly on the assay medium employed for L. gayoni 8289. This has been noted by Cheldelin and Riggs (4), and also in the present study. Both L. fermenti 9338 and L. gayoni F-20 require heated media for growth and both are classified as Type A in contrast to L. gayoni 8289 which is Type C (cf. Table VII of this thesis). According to Rogosa et al (26), L. fermenti does not require folic acid in the assay medium. However, according to Nygaard and Cheldelin (18), L. gayoni 8289 requires a relatively large supplement of folic acid. Adding to the confusion is the report of Shankman et al (27) who observed little or no requirement for folic acid by L. gayoni 8289. However, according to Kitay and Snell (14a), this latter work should be viewed with skepticism inasmuch as these workers employed excessively large inocula.

It is felt that L. gayoni 8289 is a distinct species and in view of certain interesting and unique biochemical properties its status should be clarified.