STUDIES ON THE METABOLISM OF
RIBOFLAVIN IN *ASHBYA GOSSYPH*

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I. REVIEW OF THE LITERATURE

A. The Microbiological Synthesis of Riboflavin

Riboflavin, one of the B complex vitamins, was first discovered by Goldberger and Lillie (34) while studying the nature of pellagra. They reported the existence of a heat stable substance which prevented certain deficiency symptoms in rats which were on special diets. Kuhn, et al., (49) isolated a yellow-green fluorescent substance from egg white and demonstrated that it was similar to the factor reported by Goldberger and Lillie. Ellinger and Koschura (26) reported the isolation of a similar substance from whey, liver, kidney, muscle, yeast and certain plant materials. These isolated materials were named ovoflavin, lactoflavin, heptoflavin, etc., depending on the source from which they had been isolated. When it became known that they contained the same active substance, the various names were discarded and the name, riboflavin, was adopted. Karrer, Schopp and Benz (44) described the first chemical method for the synthesis of riboflavin which has the following structure:

Von Euler, et al., (108) established that synthetic riboflavin and the natural substance were identical.

Although the chemical synthesis of riboflavin has been well established, the methods by which organisms synthesize riboflavin have not been elucidated up to the present time. Riboflavin has been reported to be synthesized in appreciable amounts by several microorganisms, namely, Clostridium acetobutylicum (117), Candida guilliermondia (90), Candida flarer (99), Mycobacterium smegmatis (53), Aspergillus flavus (70), Fusarium species (62), Saccharomyces cerevisiae (33), Eremothecium ashbyii and Ashbya gossypii (37). The closely related microorganisms, Eremothecium ashbyii (E. ashbyii) and Ashbya gossypii (A. gossypii), are used in the commercial microbiological synthesis of riboflavin due to their capacity to produce large amounts of the vitamin. For this reason, the conditions for microbiological synthesis of riboflavin have been studied more widely in these organisms than in other known producers of the compound.

The microorganisms, E. ashbyii and A. gossypii, are plant pathogens, having been isolated from the infected boll of the cotton plant. Guillermond, Fontaine and Raffy (37), in 1935, reported that the organism, E. ashbyii, produced a yellow-orange fluorescent pigment when cultured on Sabouraud's Medium at 25° C. They found that A. gossypii produced only small amounts of the pigment when cultured under the same conditions. Raffy and Fontaine (76) produced large amounts of the flavin pigment by growing E. ashbyii on Sabouraud's Agar Medium and Raffy (75) showed by rat experiments that the pigment had vitamin properties similar to riboflavin. Mirimanoff and Raffy (59) demonstrated that, microchemically, physiologically, spectrographically and fluorometrically, the flavin pigment was

identical with riboflavin.

In 1930, Farries and Bell (27) stated that A. gossypii would not grow on artificial media containing KNO_3 and ammonium salts unless supplemental growth factors were present. However, hydrolyzed natural proteinaceous material would serve as a good nitrogen source for growth. Buston and Pramanik (12) established that the factor necessary for growth was a complex. One component was inositol and the other was a substance which was similar to a then little known factor called biotin. Kogl and Fries (47) and Fries (28) showed that three factors, biotin, thiamine and inositol, were necessary for the growth of A. gossypii. Buston, Kasinathan and Wylie (11) reported that inorganic and organic ammonium salts would not give satisfactory growth when added to a medium of natural lentils extract, mineral salts and inositol. However, when β -alanine or L-asparagine was used, moderate growth was obtained.

The first systematic study of riboflavin production by E. ashbyii was made by Schopfer (89) who used a basal liquid medium containing, 1 per cent glucose, 0.1 per cent glycine or asparagine, 0.05 per cent $MgSO_4 \cdot 7H_2O$ and 0.15 per cent KH_2PO_4 , to test the effect on riboflavin synthesis when different natural extracts were added to the basal medium. Liver extract, at a concentration of 3 per cent, produced a marked stimulation of riboflavin synthesis in the basal medium. There was a considerable variation in the amount of riboflavin produced when peptones from various sources were tested. Norite treatment of peptone rendered it inactive for the synthesis of the vitamin. Biotin was reported to be essential for growth and riboflavin synthesis with thiamine and inositol acting as complementary growth factors.

Renaud and Lachaux (81, 82), in experiments with E. ashbyii grown on a liquid medium of peptone, glucose and NaCl, reported that the depth of the medium was a factor influencing yields of riboflavin, highest yields being obtained when the depth was less than 1 centimeter. They obtained highest yields on the peptone-glucose medium when 1.5 - 3.0 per cent glucose and 1.2 per cent peptone were used. The addition of 0.1 per cent $\text{NH}_4\text{H}_2\text{PO}_4$ was beneficial. Schopfer and Guilloud (91) reported that leucine or arginine, alone or in combination, supported the growth of E. ashbyii in a medium to which biotin, thiamine and inositol had been added.

Moore, de Becze and Schraffenberger (60) studied the synthesis of riboflavin by E. ashbyii using the shake flask method of growth. The optimum temperature was found to be between 26 - 30° C. with an optimum pH of 5.5 - 6.5. The pH was reported to increase to the alkaline side in the later stages of growth. Refrigeration of the culture at 4° C. for more than 7 days reduced the ability to produce riboflavin. Lyophilization destroyed the vitamin synthesizing ability completely.

Rudert (86), using E. ashbyii, reported that riboflavin could be synthesized on a carbohydrate free nutrient medium containing a metabolizable lipid, such as corn oil, egg albumin and mineral salts, as long as sufficient air was supplied to the culture. Deseive (21) observed that 0.001 per cent cystine reduced the growth of E. ashbyii and inhibited the riboflavin formation.

Chin (13) studied the growth and riboflavin formation of E. ashbyii in a medium of 1 per cent glucose, 0.2 per cent KH_2PO_4 , 0.1 per cent MgSO_4 , 0.1 per cent NaCl, 0.1 per cent agar, 5.0 per cent rice germ extract and 5.0 per cent of a suitable nitrogen source. Peptone was the best nitrogen

source, followed by glutamine, asparagine, alanine and $(\text{NH}_4)_2\text{SO}_4$ in decreasing order of suitability as a nitrogen source. Tryptophan, tyrosine, cystine, cysteine, histidine and urea were not satisfactory. Glucose, sucrose and glycerol were good carbohydrate sources but glycol, xylose and mannitol could not be used.

Phelps (63) grew E. ashbyii on a medium with solubilized casein as the nitrogen source. He reported that 0.25 - 2.5 per cent fatty acid glycerides increased the synthesis of riboflavin with the greatest stimulation being shown by butter fat.

In 1946, Wickerham, Fleckinger and Johnston (113) reported that a yellow-orange variant strain of A. gossypii resulted from continual transfers of the original light yellow strain. This new strain was able to produce appreciable quantities of riboflavin when grown under proper conditions. When grown in a medium of 0.3 per cent yeast extract, 0.5 per cent peptone and 2.0 per cent glucose at pH 6.8 - 7.0, under aerated culture conditions and at 28° C., abundant amounts of riboflavin were produced. They reported that sucrose and maltose but not lactose could be used as a substitute for glucose.

Tanner, Vojnovich and Van Lanen (100) made an extensive study of the conditions for growth and riboflavin synthesis by the organism, A. gossypii. The organism grew well over a wide temperature range but highest yields of riboflavin were obtained when the culture was incubated at 26 - 28° C. Temperatures higher than 28° C. decreased the riboflavin synthesis sharply, whereas temperatures below 26° C. caused a lesser decrease. The best initial pH range was found to be between 6.0 - 7.0. Glucose at a concentration of 0.25 - 3.0 per cent served as an adequate carbohydrate

source. Sucrose and maltose in relatively pure form could replace glucose but xylose and arabinose were not metabolized. They found that 0.5 per cent corn steep liquor with 0.5 per cent peptone, as nitrogen sources, gave very high yields of riboflavin. The fermentation was reported to be comprised of two distinct phases. In the first phase, which occurred during the first 24 - 36 hours, glucose was assimilated, the medium became more acid and very little riboflavin was formed. The second phase occurred after glucose consumption was substantially complete and was characterized by an increase in the pH of the medium and a rapid production of riboflavin.

Pridham (71) found that riboflavin synthesis by A. gossypii was increased by the addition of 1 - 2 per cent of a fermentable sugar to the medium after the fermentation had progressed for 24 - 72 hours, usually during the period of pH increase between pH 5.3 and 6.4.

Most of the early work on riboflavin synthesis by E. ashbyii and A. gossypii was mainly designed to bring about a greater commercial production of the vitamin under more economical conditions. It was not until about 1950 that any interest was directed toward how these microorganisms produced riboflavin and what chemical components were involved in this synthesis. Dulaney and Grutter (24) studied the nutritional requirements of E. ashbyii grown on a medium of glucose, hydrolyzed casein, inositol and mineral salts. They found that inositol was necessary for growth and riboflavin production and that low levels of glutamine, pantothenic acid, vitamin B₁₂, nicotinic acid, pyridoxine, choline, p-aminobenzoic acid and folic acid had no effect when added singly or together. L-proline, DL-glutamic acid and L-arginine with inositol gave growth and riboflavin

formation.

Minoura (56) reported that ashed peptone had no growth promoting not riboflavin producing effect on E. ashbyii. Hydrochloric acid hydrolyzed peptone was more effective in promoting growth and riboflavin synthesis than untreated peptone. Asparagine and arginine promoted the growth of the organism but no riboflavin was produced.

Pridham and Raper (72) gave an extensive morphological and cytological description of A. gossypii. They described the growth cycle of the organism from the vegetative stage, through the sporulation stage and to the spore germination stage. Photomicrographs illustrating the different growth phases included the "bulb forms" which accompany riboflavin synthesis. In another report, Pridham and Raper (73) also subjected A. gossypii cultures to ultra violet light, x-ray radiations and chemical mutagenic agents in an attempt to get higher riboflavin producing strains. In general, the trend of mutation effect was the formation of degenerate strains which produced less riboflavin. The addition of 0.1 per cent sodium dithionite to the stock medium enhanced pigment formation and tended to maintain the organisms at a high flavinogenic state.

Yaw (118) reported that methionine was important in the growth of E. ashbyii. Histidine was thought to be related to riboflavin synthesis either by direct incorporation into the vitamin molecule or by an indirect effect on the synthesizing system. The medium for these studies was completely defined being composed of glucose, asparagine, histidine, inositol, biotin, thiamine and mineral salts.

MacLaren (50) studied the effect of various purines and pyrimidines on the growth and riboflavin synthesis of E. ashbyii in a variation of

Yaw's medium. The purines, adenine, xanthine and guanine increased riboflavin synthesis without increasing the growth of the organism. The pyrimidines, uracil and thymine, did not effect the growth of the organism but uracil inhibited the synthesis of riboflavin. MacLaren postulated that a "purine pathway" may exist in the process of riboflavin biosynthesis.

Dikanshaya (22, 23) observed that more active growth of E. ashbyii produced less riboflavin. Folic acid and p-aminobenzoic acid were needed for growth and biotin and inositol were needed for riboflavin synthesis. A low vitamin content of the medium along with sparse mycelial growth contributed to favorable conditions for riboflavin formation. Strains of E. ashbyii most effective in riboflavin synthesis had weaker cytochrome systems but more active dehydrogenase and proteolytic enzyme systems than low riboflavin producing strains.

Minoura (57, 58) reported that arginine was most effective in promoting riboflavin synthesis in E. ashbyii. Other amino acids, in decreasing order of effectiveness were, aspartic acid, glutamic acid, asparagine, proline, lysine, methionine, phenylalanine, leucine and tyrosine. Serine and norleucine were inferior to $(\text{NH}_4)_2\text{SO}_4$. Asparagine was most effective in increasing growth. The addition of 0.01 - 0.1 milligrams of γ -benzene hexachloride per 100 milliliters of medium increased the synthesis of riboflavin; however, concentrations greater than 0.1 milligram were inhibitory to the vitamin synthesis.

Van Lanen, Smiley and Stone (105) devised an amino acid medium for the growth and riboflavin synthesis of A. gossypii. Glycine, alanine, threonine and proline added to a sucrose, vitamin, mineral salts medium

gave a noticeable yield of riboflavin. Dicarboxylic acids and related compounds were reported to interfere with riboflavin synthesis.

Goodwin and Pendlington (35), using a 0.005 per cent peptone basal medium, reported that L-serine, L-threonine or L-tyrosine stimulated riboflavin synthesis in E. ashbyii. L-glutamic acid, L-aspartic acid and L-asparagine stimulated both growth and vitamin synthesis, whereas L-cystine inhibited both. The other amino acids and $(\text{NH}_4)_2\text{SO}_4$ were without effect. Xanthine, adenine and adenosine increased riboflavin synthesis but pyrimidines had no effect. Alloxan inhibited growth and riboflavin synthesis.

Klungsoyr (45, 46) obtained riboflavin synthesis by cells of E. ashbyii suspended in a buffer composed of NaCl, HCl, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, Na_2HPO_4 adjusted to pH 6.9. He proposed that the riboflavin was synthesized in preformed cells and that growth and riboflavin synthesis may be regarded as independent processes. When acetic acid- 1-C^{14} was added to the synthesizing cells, less than 0.01 per cent of the added C^{14} activity was recovered in the riboflavin and this was found to be in the carbon-2 atom of the ring portion of the molecule. The activity from added C^{14} -formate was also found to be in the carbon-2 atom of the ring.

McNutt (51, 52) continued the work of MacLaren on E. ashbyii, using uniformly labeled adenine. He reported that the adenine was principally incorporated into the 6,7-dimethylisalloxazine portion of the riboflavin molecule, further substantiating the postulated "purine pathway" of riboflavin synthesis. Naturally occurring purine nucleosides and nucleotides were no more effective than the parent purines in increasing riboflavin synthesis.

Brown, Goodwin and Pendlington (10) reported that pyruvate was the

only non-nitrogen compound related to serine and threonine which gave an increase in riboflavin formation with E. ashbyii. Aminopterin, at a concentration of 2 micrograms per 100 milliliters inhibited the growth of the organism but stimulated flavogenesis.

Plaut (66, 67, 68) reported on the location of tagged atoms in the riboflavin molecule when C^{14} -formate, C^{14} -bicarbonate, acetic acids- $1-C^{14}$ and $2-C^{14}$, glycines- $1-C^{14}$ and $2-C^{14}$, and totally labeled glucose were added to a growing culture of A. gossypii in a medium of corn steep liquor, peptone and glucose. He found that the C^{14} -formate activity was concentrated in the carbon-2 atom of the riboflavin, whereas the C^{14} -bicarbonate activity was in the carbon-4 atom of the molecule. Acetic acid- $1-C^{14}$ produced activity in the carbon-4 atom but to a lesser degree than C^{14} -bicarbonate. The acetic acid- $2-C^{14}$ activity was in the methyl groups, carbons-5,6,7,8,8a and 10a and in the ribityl side chain of the molecule. A similar distribution of activity was shown with uniformly labeled glucose. The activity of the labeled glycines was located predominantly in carbons-4a and 9a of the ring portion of the riboflavin molecule. The addition of glucose- $1-C^{14}$ and glucose- $6-C^{14}$ resulted in the activity being primarily located in the methyl groups and carbons-5 and 8. Plaut expressed the possibility of a two carbon fragment being involved in the synthesis of the cyclic carbon ring of the riboflavin molecule. Plaut and Broberg (69) have reported further studies on the incorporation of C^{14} -acetate and glucose into the ribityl side chain of riboflavin.

The abundance of research on the problem of riboflavin synthesis by microorganisms is shown by the number of reports in the literature, however, the question of the nature of the pathway of synthesis remains

unanswered. Further research on the growth and metabolism of these riboflavin synthesizing organisms will no doubt eventually provide the answer to this question.

B. The Biosynthetic Relationships of Amino Acids

Amino acids are often referred to as the "building blocks" of protein, but they are also essential in the biosynthesis of other important nitrogen containing biological compounds. The structure of riboflavin indicates that there are four nitrogen atoms per molecule and, therefore, amino acids might be expected to play a key role in the biosynthesis of the molecule. The relationship of amino acids to riboflavin biosynthesis is emphasized by the fact that formation of the vitamin by microorganisms is dependent on a suitable organic nitrogen source as shown by many references in the preceding section. The review of the biosynthetic relationships of amino acids in this section will be of general nature and, for the most part, restricted to processes in microorganisms. A more comprehensive survey of the subject may be obtained from recent reviews (19, 54).

One of the important amino acids involved in transamination reactions is α -alanine which is present in a wide variety of natural proteinaceous materials. It is related to pyruvic acid through the transamination reaction sequence, glutamate + pyruvate $\longleftrightarrow$ α -ketoglutarate + α -alanine, which is reversible (36). This reaction serves as the mechanism for the microbiological synthesis and degradation of α -alanine.

The compound, β -alanine, is one of the less common amino acids but it is important as a part of the vitamin, pantothenic acid which makes up part of coenzyme A. Gale (31) reported that β -alanine could be formed

by the decarboxylation of aspartic acid. It has been shown by Roberts, et al., (83) that β -alanine was associated with transamination in Aspergillus fumigatus; however, the reaction mechanism has not been established. In rat feeding experiments with isotopic compounds, Phil and Fritsson (65) found that β -alanine was rapidly oxidized and part of the carbon chain converted to acetic acid. They were of the opinion that oxidation took place after the amino acid had been deaminated and decarboxylated.

Aspartic acid, like α -alanine, is important in nitrogen transfer reactions involving transamination. It is synthesized in many microorganisms by the amination of fumaric acid which is a reversible reaction catalyzed by the enzyme aspartase (30). It has also been reported to be formed by a transamination reaction involving oxaloacetic acid (48). Abelson and Vogel (3) stated that aspartic acid was the precursor for the amino acids, homoserine, methionine, threonine and isoleucine, in Torulopsis utilis and Neurospora crassa. Aspartic acid has been reported to be associated with the biosynthesis of Coenzyme A (31), purines (110) and pyrimidines (77). Asparagine is formed by the amidation of aspartic acid; however, the reaction mechanism is not known. Black and Gray (7) reported that β -aspartyl phosphate may be a precursor of asparagine. In a rat liver preparation, Meister and Fraser (55) found that asparagine could be formed by the transamination of α -ketosuccinamic acid with glutamine which was the most active amino group donor. Asparagine is important as a nitrogen source for many microorganisms.

Another amino acid, glutamic acid, occupies an important position in the nitrogen metabolism of microorganisms and animals. It is primarily

formed through the transamination of α -ketoglutaric acid (32) and the reaction of α -ketoglutarate with ammonia catalyzed by the enzyme glutamic dehydrogenase. Glutamic acid can also result from the degradation of proline, arginine or histidine. Shemin and Russel (92) reported the biosynthesis of glutamate by the reaction of glycine and succinate via δ -aminolevulinic acid. Abelson (1), using isotope inhibition analysis, claimed that glutamic acid was the precursor of proline, ornithine, citrulline and arginine. Glutamine, the amide of glutamic acid, is synthesized by the combination of glutamic acid and ammonia in the presence of ATP (42, 112).

Arginine is synthesized from glutamic acid, via ornithine and citrulline, in E. coli and many other microorganisms. Anderson-Kotte, et al., (5) established that in E. coli N-acetylglutamic acid was one of the intermediates in the synthesis of ornithine from glutamate. Srb and Horowitz (95) reported the formation of citrulline from ornithine in Neurospora, the mechanism not being known. Citrulline and aspartic acid form arginine via the intermediate, arginino-succinate (79, 78). Vogel, et al., (106) reported that the activity of labeled N-acetylglutamic acid was heavily concentrated in the arginine synthesized by E. coli, verifying the glutamate to arginine sequence.

Vogel and Davis (107) have reported that proline, an imino acid, was formed from glutamic acid via glutamic γ -semialdehyde and Δ^1 -pyrroline-5-carboxylic acid in two mutant strains of E. coli.

The biosynthesis of lysine is believed to follow different pathways in bacteria and fungi. Davis (17) reported that in E. coli α,α' -diaminopimelic acid is an intermediate in the synthesis of lysine from aspartic

acid. In Neurospora, Windsor (114) showed that α -aminoadipic acid could be converted to lysine. Strassman and Weinhouse (97) established that the alpha carboxyl carbon of lysine was derived from acetate as shown by the use of labeled acetate as the sole carbon source for a yeast and Neurospora. They proposed that carbons- 3 and 6 resembled the succinyl moiety of α -ketoglutarate and that acetate probably condensed with succinyl-Coenzyme A in the initial step of lysine biosynthesis. Rothstein and Millar (85), using lysine-6- C^{14} proposed that lysine degraded to α -ketoglutaric acid and acetate via α -aminoadipic acid.

The biosynthesis of histidine is not too clearly understood at the present time. Purine bases have been reported to be related to histidine biosynthesis in lactic acid bacteria (9). Fries (29) suggested that asparagine may function as a nitrogen donor in some step of histidine biosynthesis since either histidine or asparagine were required by a mutant of the mold, Ophiostoma. In accumulation studies with Neurospora, Haas, et al., (38) have proposed a path of synthesis via imidazole glycerol phosphate, imidazole acetol phosphate, histidinol phosphate, histidinol and histidine. In yeasts grown with labeled acetate, the carboxyl group of histidine was reported to be derived from the methyl group of acetate (25). Ames and Mitchell (4) have suggested that the five carbon chain of histidine might come from pentose-5-phosphate. Using isotope distribution analysis, Wolf (115) showed that histidine could be degraded to glutamic acid through some unknown path of reactions.

Tryptophan, phenylalanine and tyrosine are biosynthetically related through the common precursor, shikimic acid (16). Davis (18) reported that phenylalanine was synthesized from shikimic acid via prephenic acid and

phenylpyruvic acid. Prephenic acid has also been shown to be a precursor for tyrosine biosynthesis in a tyrosine requiring mutant of Neurospora (20). Srinivasan, et al., (96), in experiments with labeled glucose, have suggested that the side chains of phenylalanine and tyrosine may arise from a glycolysis product. In tryptophan biosynthesis, shikimic acid was converted to anthranilic acid (102). Rafelson, et al., (74) showed that acetate was added to anthranilic acid to give indole. Tatum and Shemin (101) reported that serine combined with indole to form tryptophan in the presence of the enzyme, tryptophan desmolase.

Serine and glycine are known to be related in their metabolisms but there is some question whether serine or glycine is formed first. Ehrensverd, et al., (25) reported that the pathway, pyruvate, serine to glycine, probably existed in Torulopsis utilis, since evidence indicated that the same isotopic distribution in alanine, serine and glycine occurred when labeled acetate was used as the carbon source. Abelson (1) also reported the synthesis of glycine from serine in E. coli using labeled serine and glycine in the presence of glucose. Wang, et al., (111) obtained evidence that serine was synthesized from glycine in baker's yeast when pyruvate-2- C^{14} was the sole carbon source. Low (43) has presented evidence that the carboxyl group of glycine is incorporated into the pentoses of ribonucleic acids. Glycine has also been shown to be incorporated into purine bases (98).

Cystine and cysteine are two sulfur containing amino acids which are important in metabolism. Abelson (1) reported that inorganic sulfur and serine combined to form cysteine which was further converted to cystine in E. coli. Pierpoint and Hughes (64) have pointed out that in

Lactobacillus arabinosus Coenzyme A formation from pantothenic acid would proceed only if cystine was present in the medium.

Ehrensward, et al., (25) proposed that threonine synthesis originated with aspartic acid in Torulopsis utilis and E. coli since the isotope distribution with labeled acetate was the same in aspartic acid and threonine. Woods, et al., (116) have reported that threonine can spare aspartic acid requirements in certain lactic acid bacteria. The work of Abelson, et al., (2) has shown that homoserine can serve as a precursor for threonine. Black and Wright (8) have suggested that β -aspartyl phosphate and aspartic acid β -semialdehyde may be intermediates between aspartic acid and homoserine in the pathway of threonine synthesis. The formation of homoserine from β -aspartyl phosphate has been shown to be inhibited by glutamic acid in Leuconostoc dextranicum (80).

The biosynthesis of valine has been shown to proceed from pyruvic acid via α -ketoisovaleric acid (1, 3). Abelson (1) has suggested that leucine may be derived from pyruvic acid via α -ketoisovaleric acid and α -ketoisocaproic acid. He proposed that α -ketoisovalerate may contribute carbons- 3 and 6 to combine with acetate in the formation of α -ketoisocaproic acid. Ehrensward, et al., (25) and Outinelli, et al., (15) have established that the α -carbon atom and carboxyl group of leucine are derived directly from acetate in yeasts and bacteria. Abelson and Vogel (3) reported that isoleucine is formed from threonine via the intermediates α -ketobutyric acid and α -keto- β -methylvaleric acid.

Methionine is a sulfur containing amino acid which is important in transmethylation reactions. Homoserine and cysteine combine to form cystathionine which is converted to methionine (40, 41). Cystine can spare but

not replace methionine as a growth factor (84).

The relationships of amino acids are summarized in Figure 1.

C. Chromatography

Chromatography is an important technique which is used in the separation and analysis of biological materials. The first application of chromatography is usually attributed to Tswett (103) who separated green leaf extract into different fractions by means of a column of powdered calcium carbonate. The introduction of paper-partition chromatography by Consden, Gordon and Martin (14) in 1944 was one of the most important recent advances in chromatography. Paper chromatography is probably one of the most widely used methods in the biological research of today.

There have been many variations of the original technique which resolved mixtures on paper strips or sheets by means of various solvents. Rutter (87, 88) introduced a method which he called circular chromatography, which allowed a separation of a mixture by the movement of the solvent system through a small wick cut from the center of a filter paper disk, thus giving a series of rings making up the components of the mixture. The method gives the advantage of rapid separation of mixtures but the resolution is not very clear cut.

The solvent system is important for the proper separation of components in paper chromatography. One of the first solvents was composed of water saturated phenol solution (14). Slotta and Primosigh (93) introduced the solvent system, n-butanol-acetic acid-water, which is widely used for the separation of many groups of compounds. The components of the solvent were mixed in the ratio of 4:1:5, respectively, by volume and

THE BIOSYNTHETIC RELATIONSHIPS OF AMINO ACIDS

Figure 1

the top layer used as the solvent. Another useful solvent is composed of pyridine, tertiary amyl alcohol and water in the ratio of 3.5:3.5:3 by volume (61). A comprehensive survey of the voluminous literature on chromatography is beyond the scope of this review. The references presented include only those considered to be pertinent to the problem.

D. Riboflavin Assay

After riboflavin was established as a growth factor, it was necessary to provide a means of analyzing for the vitamin. Von Euler and Malmberg (109) introduced the biological assay method which was designed to measure riboflavin by comparing changes in growth rates of test animals, usually rats, treated with the unknown sample and animals given standard amounts of riboflavin.

Snell and Strong (94) developed the microbiological method of riboflavin assay which is extensively used today. It is based on the growth response to riboflavin by the microorganism, Lactobacillus casei, which produces lactic acid in proportion to the amount of the vitamin present under certain conditions. The lactic acid produced with the unknown is titrated and compared with that produced with standard amounts of the pure riboflavin. The assay is very reliable but it must be carried out under carefully controlled conditions and requires the preparation of special culture media.

The fluorometric analysis of riboflavin was introduced by Van Eckelen and Emmerie (104). Under the influence of ultraviolet light, riboflavin exhibits a characteristic fluorescence which is proportional in intensity to the amount of riboflavin in dilute solutions. Hodson and

Norris (39) modified the method by introducing the use of sodium dithionite to reduce the riboflavin to the colorless form without effecting the other fluorescing substances present, thus increasing the accuracy of the procedure. The Association of Vitamin Chemists (6) has described a standard fluorometric procedure, including extraction techniques and methods of performing the assay.

Tanner, et al., (100) extracted the riboflavin from A. gossypii cells by autoclaving them for 30 minutes at 15 pounds steam pressure in a 0.123 M sodium acetate buffer at pH 4.7.

Yaw (118) used a colorimetric assay method to determine riboflavin extracted from E. ashbyii cells. The method, using a Klett-Summerson colorimeter, was reported to agree within plus or minus 5 per cent of the microbiological method; however, there were no details given for the procedure.

II. STATEMENT OF THE PROBLEM

Although the conditions for the commercial synthesis of riboflavin are well established, the mechanisms by which microorganisms synthesize it have not been fully elucidated. This study of the metabolism of riboflavin by Ashbya gossypii was undertaken in an effort to gain more facts pertaining to the mechanism of riboflavin synthesis in microorganisms and, thereby, bring about a more complete understanding of the pathway and intermediates participating in the synthesis.

III. EXPERIMENTAL

A. Apparatus

1. The colorimeter. An Evelyn Photoelectric Colorimeter, manufactured by the Rubicon Company of Philadelphia, Pennsylvania, was the instrument used in turbidimetric measurements of growth and colorimetric determinations of riboflavin. The instrument is a single-photocell, direct reading, photoelectric photometer equipped with light filters and a light beam galvanometer.

2. The shaker. A platform reciprocating shaker, constructed in the Chemistry Department shop at the University of Florida, was used to maintain an efficient aeration of the cultures during incubation in liquid media. The shaking platform accommodated fifty 250 ml. narrow mouth Erlenmeyer flasks which were held in place by a removable plywood top containing 50 two and one-half inch holes to separate the flasks. A second layer of fifty flasks could be added by attaching a special tray to the main shaking platform. The shaking rate could be adjusted by using different size pulleys; however, the size pulley used provided a shaking rate of 90 three inch strokes per minute. The shaker was housed in a constant temperature room at 28° C.

3. The pH meter. A Beckman Model H-2, line operated, glass electrode pH meter with attached constant voltage transformer was used for all pH measurements. The pH meter was standardized before use against a pH 7.0 buffer prepared from a Beckman concentrated liquid buffer. The

instrument was allowed to warm up for at least a half an hour before standardization.

4. The fluorometer. A Coleman Universal Spectrophotometer, Model 14, was used for the fluorometric determinations of riboflavin. The instrument was adapted to fluorometric analysis with a Universal Ultraviolet Illuminator and a set of filters, UV-2 and PC-2, specific for riboflavin analysis. Round, fluorometer microcuvettes which held 10 ml. of sample were used with a special microfluorometric cuvette jig.

B. General Procedures

1. Maintenance of the culture. The culture of A. gossypii, strain NRRL Y-1056, obtained from the Northern Regional Research Laboratory of the Department of Agriculture in Peoria, Illinois, was maintained on stock agar slants which were prepared from a medium containing 2.0 per cent glucose, 1.0 per cent Difco-peptone, 0.5 per cent Difco-yeast extract and 1.8 per cent Difco-agar. The medium was sterilized by autoclaving at 15 pounds steam pressure for 30 minutes and slanted. Transfers were made to new slants every 48 hours in order to maintain an actively pigmenting culture. Every two or three weeks, a 4-day-old, pigmenting culture was placed in the refrigerator at 8 - 10° C. so that a pigmenting strain would be available in case of contamination or loss of the ability to produce constant pigmentation. The cultures were incubated in a constant temperature room at 28° C.

2. Experimental media. Liquid media were prepared by adding different nitrogen sources at different concentrations to a general basal

medium containing 2.0 g. glucose, 0.1 g. KH_2PO_4 , 0.1 g. K_2HPO_4 , 0.1 g. NaCl, 0.05 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.0 mg. inositol, 0.1 mg. thiamine-HCl and 2.0 ug biotin in 100 ml. of distilled water. In the early experiments, 0.05 g. NaCl was used in place of the 0.1 g. NaCl as shown in the general basal medium. All media were adjusted to pH 6.5 - 6.6 with 0.1 - 1.0 molar solutions of either NaOH or KOH unless otherwise specified. In each shaker flask for inoculation, 20 ml. of liquid medium was placed. When solid media were used in an experiment, 1.8 g. of Difco-agar was added to each 100 ml. of liquid media. Such solid media were dispensed in 10 to 12 ml. volumes per tube and slanted after autoclaving. All media were sterilized by autoclaving at 15 pounds steam pressure for 17 minutes.

3. Inoculation and incubation of media. The inoculum for the shaker flasks was prepared from cells of A. gossypii grown on stock agar slants for 24 to 72 hours depending upon the experimental conditions being studied. In the early experiments, the inoculum was prepared by washing the cells from each slant with approximately 10 ml. of sterile distilled water and breaking up clumps with the inoculation loop. This cell suspension was transferred to a sterile, 15 ml. centrifuge tube, centrifuged, washed twice with 10 ml. of fresh sterile distilled water and resuspended in 10 - 12 ml. of sterile distilled water. A volume of 0.2 - 0.3 ml. of the washed cell suspension was used to inoculate each shaker flask. In later experiments, the suspension, before being washed, was shaken with sterile glass beads to provide a more uniform inoculum.

The flasks of inoculated experimental media were incubated on the

shaker in a constant temperature room at 28° C. for a given period of time, in most cases, for 96 hours. Solid media slants were incubated at the same temperature as the flasks for the desired length of time.

4. Measurement of growth. Growth was measured by turbidimetric measurements or by obtaining the weight of the dry cells. Turbidimetric measurements were carried out in the Evelyn photoelectric colorimeter using the 540 millimicron green filter. The instrument was set at 100 per cent transmittance with a distilled water blank and the value of an air blank was determined with this setting. Further adjustments of the instrument were made by setting the instrument on the air blank reading throughout each set of determinations. After incubation each culture was adjusted to the original volume of 20 ml. with distilled water and then added to a colorimeter tube which was placed in the colorimeter. The growth was measured as per cent transmittance which varied inversely as the amount of growth present. The readings were made within a few seconds after the suspensions were prepared because the cells tended to pack to the bottom of the tube. The method was rapid and reasonably accurate at lower concentrations of cells, however, at higher concentrations of cells it was difficult to measure differences in growth.

The determination of the dry weight of cells was accomplished as follows: the medium containing the cells was diluted to the original volume of 20 ml. After thorough mixing, a 10 ml. aliquot of a suspension was removed for riboflavin determination and the remaining portion of the suspension was suction filtered onto a previously dried and weighed filter paper. After the cells and filter paper were washed with distilled

water, they were placed in an oven at 105° C. to dry for 12 hours. The dried cells and paper were reweighed and the dry weight of cells per 10 ml. of aliquot was determined by difference. The dry weight method provided a more accurate means of measuring growth than the turbidimetric method.

C. Riboflavin Determination

1. Preparation of the sample for riboflavin determination. The riboflavin was extracted from the cells of A. gossypii by autoclaving them for 30 minutes at 15 pounds steam pressure. Before extraction, 2.5 ml. of 0.123 M sodium acetate buffer, at pH 4.7, was added to each 10 ml. of the growth medium containing cells. The buffer was added to stabilize the riboflavin during extraction, since it would be rapidly destroyed in near neutral or alkaline solutions. The autoclaved mixture was filtered to remove the cells and the filtrate was diluted to an appropriate volume, usually 25 ml., with distilled water. The necessary dilutions were made from this solution before the riboflavin determinations were made.

2. Fluorometric determination of riboflavin. The fluorometric determination of riboflavin described by the Association of Vitamin Chemists (6) was modified to adapt it for use with the Universal spectrophotometer. Fluorescence was measured by readings on the per cent transmittance scale of the instrument. The standard solutions were made from a standard stock solution which was prepared by dissolving 25 mg. of riboflavin in 1000 ml. of a solution containing 2 ml. glacial acetic acid. Ten milliliters of each diluted unknown riboflavin solution and of the

standard solution were added to separate test tubes each of which contained 1 ml. of glacial acetic acid. After thoroughly mixing the solutions, each solution was transferred to a special microcuvette and fluorescence measurements were made. Duplicate determinations were made for each unknown or standard riboflavin solution. The cuvettes were rinsed thoroughly with distilled water and dried after each determination.

The fluorometric determination of riboflavin was made on a specially adapted Universal Spectrophotometer. The instrument was adjusted to zero per cent transmittance using a water blank, then using a solution containing 50 ug. of sodium fluorescein per liter of distilled water as a substitute standard, the Bal dial was set at a per cent transmittance for reference, usually between 40 to 60 per cent, and then the index of the galvanometer scale was adjusted to read zero with the fluorometer knob of the instrument. During the assay, frequent checks were made with the sodium fluorescein solution by readjusting the instrument to the reference transmission selected in order to maintain standardized conditions. A water-dithionite blank value was determined on a solution of 10 ml. of distilled water and 1 ml. of glacial acetic acid containing approximately 20 mg. of sodium dithionite.

After the fluorescence of a riboflavin sample was measured, approximately 20 mg. of sodium dithionite was added to the sample in the cuvette and thoroughly mixed. The dithionite reduced the riboflavin to the colorless form and the sample was again measured to determine the fluorescence by substances other than riboflavin in the reduced sample. The water-dithionite blank value was subtracted from the fluorescence of the reduced riboflavin sample to give the correction for the fluorescence

of non-riboflavin substances present in the untreated sample. The fluorescence due only to riboflavin in the sample was determined by subtracting the correction for the non-riboflavin fluorescence from the fluorescence of the untreated sample. A series of standard riboflavin solutions were measured in order to calibrate the instrument and to prepare a standard curve. The standard stock solution was diluted to give duplicate solutions ranging in concentration from 0 ug. to 1 ug. of riboflavin per ml. The results of a standard calibration determination are shown in Table 1 and the data plotted as a standard curve in Figure 2.

TABLE 1

THE FLUORESCENCE OF STANDARD RIBOFLAVIN SOLUTIONS

Riboflavin Solutions (ug/ml)	Fluorescence (Dial Readings)			Riboflavin	Average
	Untreated	Reduced	Correction ^{**}		
0.05	76.2	73.9	0.1	76.1	
0.05	76.6	73.8	0.0	76.6	76.3
0.10	79.2	73.9	0.1	79.1	
0.10	79.1	74.0	0.2	78.9	79.0
0.30	89.1	73.9	0.1	89.0	
0.30	89.0	74.0	0.2	88.8	88.9
0.50	98.0	73.9	0.1	97.9	
0.50	98.2	74.0	0.2	98.0	98.0
0.75	108.7	73.9	0.1	108.6	
0.75	109.2	74.0	0.2	109.0	108.8
1.0	119.6	73.9	0.1	119.5	
1.0	119.8	74.0	0.2	119.6	119.6

* - Average water-dithionite blank value 73.8.

** - Correction for non-riboflavin fluorescence.

FIGURE 2

A STANDARD RIBOFLAVIN CURVE

Since the curve in Figure 2 showed that the most desirable concentrations of riboflavin for the determination were between 0.3 ug. and 1.0 ug. per ml., dilutions of unknown samples were made to give concentrations which would fall within this desired range. The unknown samples were treated in the same manner as the standard samples above, fluorescence readings were made and amounts of riboflavin were read from the standard curve prepared for each series of unknown determinations. Although the fluorometric method gave good results, it required 4 to 6 hours to make one complete assay of a series of samples, therefore, it placed a limit on the number of experiments which could be performed at one time.

D. Chromatography

1. Paper sheets and strips. The conventional methods of ascending paper chromatography were employed with sheets or strips of Whatman No. 1 filter paper. The butanol-acetic acid-water and the pyridine-tert. amyl alcohol-water solvents were used as the resolving systems. Small sheets, 18 cm. by 18 cm., were formed into cylinders and run in large mouth jars. The cylinder was held in form by a nichrome wire shaped in the form of a small crescent. The spots were developed by ultraviolet light, ninhydrin solution or other standard developing solution. The small two-dimensional chromatograms could be completed in 7 to 8 hours as compared to at least 48 hours for the standard large sheets.

2. Circular chromatography. The technique of circular chromatography was employed as a rapid means of chromatographic separations and for the identification of component fractions present in a solution.

Circles of Whatman No. 1 filter paper, 20 cm. in diameter, were cut into quarters and a wick, approximately 2 mm. wide, was cut from the arc side of the quarter to the center of the quarter. The solution, to be chromatographed, was applied at the bend where the wick and the paper connected. As in all paper chromatograms, the smaller the spot of application, the better the resolution of the components. The chromatographic chamber was made of two 9 cm. fluted watch glasses, one to serve as the solvent reservoir and the other as the top. After 5 to 6 ml. of solvent had been added to the reservoir, the wick was bent down and placed in the solvent with the remainder of the paper resting on the watch glass. The watch glass top was put in place and the chromatogram allowed to flow as a circular migration from the wick in the center of the paper. The chromatogram was removed from the solvent when the solvent front had progressed 25 to 30 mm. from the point of application. The solvent front was marked and the paper dried, sprayed and developed according to standard procedures. The R_f values of the ring spots were measured in the conventional manner by dividing the ring distance by the solvent front distance.

Solutions of amino acids and peptides, prepared by adding 50 mg. of the individual amino acid or peptide to 100 ml. of distilled water were chromatographed. In Table 2, the R_f values of amino acids and peptides are recorded for circular chromatograms using the solvent systems, n-butanol-acetic acid-water and pyridine-tertiary amyl alcohol-water. The ring spots were developed with a spray solution of ninhydrin.

3. Column chromatography. Column chromatography was used in the separation of peptone into different fractions. The column was made of a standard 50 ml. burette, however, a pyrex glass condenser tube, 250 mm.

TABLE 2

THE R_f VALUES OF AMINO ACIDS AND PEPTIDES IN BUTANOL-ACETIC ACID-WATER AND PYRIDINE-TERTIARY AMYL ALCOHOL-WATER SOLVENTS ON CIRCULAR CHROMATOGRAMS

Amino Acids and Peptides	R_f Values*		Ninhydrin Color
	BuOH-HAc-Water	Pyridine-t-amyl alcohol-Water	
alpha-Alanine	0.47	0.50	Red-purple
beta-Alanine	0.43	0.44	Purple-blue
Arginine-HCl	0.34	0.34	Purple
Asparagine	0.39	0.38	Brown
Aspartic Acid	0.38	0.37	Purple
Cystine-HCl	0.33	0.31	Purple
Glutamic Acid	0.50	0.41	Red-purple
Glycine	0.41	0.43	Purple
Histidine-HCl	0.36	0.44	Blue-purple
Isoleucine	0.74	0.70	Purple
Leucine	0.77	0.70	Purple
Lysine-HCl	0.38	0.31	Red-purple
Methionine	0.64	0.66	Purple
Phenylalanine	0.72	0.71	Purple
Proline	0.51	0.54	Yellow
Serine	0.44	0.46	Purple
Threonine	0.50	0.51	Red-purple
Tryptophane	0.68	0.69	Purple
Tyrosine	0.60	0.66	Red-purple
Valine	0.64	0.61	Red-purple
Alanylphenylalanine	0.77	0.70	Brown, turns pink
Glycylglycine	0.44	0.36	Brown, turns pink
Glycylalanine	0.70	0.61	Brown, turns pink

* - Average of duplicate measurements.

by 10 mm., can be used when fitted with a removable stopcock at the narrow end. Approximately one-half inch of glass wool was packed into the bottom of the column to retain the material forming the stationary phase of the column. In the experiments with peptone, Whatman powdered cellulose was

used as the stationary phase. Approximately 8 grams of powdered cellulose was suspended in the butanol-acetic acid-water solvent and carefully added to the column. The column was allowed to pack by the flow of the solvent through the cellulose, taking care to tap the air bubbles out as the column formed. It is important to keep the solvent level above the top of the cellulose since once the cellulose becomes free of the solvent, a pocket of air forms thus destroying the uniformity of the stationary phase. After all of the cellulose was packed into the column it was washed with 50 to 60 ml. of fresh solvent. The solvent was allowed to run through the column until it was about 1 cm. from the top of the cellulose and at this point the stopcock was shut off.

The peptone was prepared by dissolving about 1.0 g. of Difco-peptone in a minimum amount of water. The peptone solution was mixed with powdered cellulose to form a paste which was dried at 75° C. for about 30 minutes. The peptone-cellulose mixture was added to the top of the cellulose column and carefully packed. Fresh solvent was run into the column and the stopcock opened to allow a flow of the solvent to pass through the cellulose. Samples of the effluent from the column were checked periodically by wetting a filter paper strip, drying it and spraying it with ninhydrin solution to show the appearance of the ninhydrin positive portions of peptone. After the first indication of ninhydrin positive material in the effluent, fractions were collected from the column every 3 to 4 ml. Circular chromatograms were made of each fraction as it was collected which provided an indication of the progress of the separation on the column. This speeded up the entire procedure, so that similar fractions could be combined as the separation was taking place. This application of circular chromatography

served to stress the utility of the technique in cases where a rapid chromatographic interpretation of mixtures is desired.

E. Experiments

1. The Relationship of Riboflavin Synthesis to Growth in a Peptone Medium. The work of Tanner, et al., (100) with Ashbya gossypii has shown that riboflavin synthesis did not begin until after the organism had grown for approximately 48 hours. They suggested that riboflavin may be synthesized only after the organism had reached a certain stage of growth but gave no data to indicate at what stage of growth the synthesis started. This experiment was undertaken to show the relationship of riboflavin synthesis to the growth of A. gossypii.

A liquid medium was prepared which contained 2.0 per cent glucose, 0.5 per cent peptone, 0.1 per cent KH_2PO_4 , 0.1 per cent K_2HPO_4 , 0.05 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.05 per cent NaCl , in 100 ml. distilled water. The medium was adjusted to pH 6.5 and autoclaved in 250 ml. Erlenmeyer flasks. Twenty flasks containing the sterilized medium were inoculated with a 48 hour washed cell suspension of A. gossypii in the usual manner. Two flasks were used for the duplicate zero time determinations. The rest of the flasks were incubated on the shaker. Two flasks were removed from the shaker after each of the following time intervals: 8, 12, 18.5, 24, 29, 36, 48, 72 and 96 hours. The contents of each flask were diluted to 20 ml., the original volume, and transferred to a colorimeter tube and the turbidity determined by measuring the per cent transmittance. The pH of the contents of each tube was determined next, followed by the addition of 5 ml. of acetate buffer and extraction of the riboflavin. The extracted cells were

filtered onto previously weighed filter papers and dried for dry weight determinations of the extracted cells. Each filtrate was removed and diluted to the appropriate concentration range and the riboflavin was determined fluorometrically. The results of the experiment are given in Table 3 and are shown graphically in Figure 3.

The growth of A. gossypii follows a pattern typical of most microorganisms, as shown by the turbidometric growth curve in Figure 3. After a 6 hour lag period, the growth increases rapidly for a 10 to 12 hour period which corresponds to the logarithmic phase of growth. At this phase, growth slowly begins to level off and reaches a maximum between 38 and 45 hours. The growth, as determined by the dry weights of extracted cells, follows a pattern similar to that determined turbidimetrically, reaching a maximum dry weight at 36 hours. The dry weights of extracted cells decrease rapidly from 36 hours to the final determination at 96 hours, which is approximately 54 per cent of the maximum dry weight. This probably indicates a greater disruption of the cells during the extraction procedure with the resultant loss of intracellular materials. This is to be expected since older cells are known to be more easily affected by physical environment. In the work of Mac Laren on E. ashbyii, the dry weight of extracted mycelia was used as an indication of growth for comparing the effects of various purines and pyrimidines in different media. On the basis of the experiment just discussed, there may be some question whether the dry weights of extracted cells could be validly used as a true measure of growth. A better indication of the true growth of the organism might be obtained by taking the dry weights of an aliquot of cells before the extraction of riboflavin.

TABLE 3

THE PER CENT TRANSMITTANCE, pH AND RIBOFLAVIN CONTENT OF CULTURES OF
A. GOSSYPII, AND DRY WEIGHTS OF THEIR EXTRACTED CELLS,
 AT DIFFERENT AGES GROWN IN A PEPTONE MEDIUM

Age (hrs)	Transmittance (%)	pH	Dry Wt. Cells (mg)	Riboflavin		
				Aliquot (ug/ml)	Dilution Factor	Total (ug/ml)
0	97.8	6.50	0.6	0.02	2.5	0.05
	98.1	6.50	1.1	0.03	2.5	0.08
Ave.	97.9	6.50	0.9			0.07
8	93.0	6.30	1.7	0.04	2.5	0.10
	93.5	6.35	1.5	0.04	2.5	0.10
Ave.	93.3	6.33	1.6			0.10
12	72.0	6.00	7.5	0.06	2.5	0.15
	70.2	5.90	6.2	0.08	2.5	0.20
Ave.	71.1	5.95	6.9			0.17
18.5	23.0	6.00	37.6	0.13	5.0	0.65
	22.1	5.90	42.0	0.15	5.0	0.75
Ave.	22.6	5.95	39.8			0.70
24	14.0	5.65	66.5	0.16	5.0	0.80
	13.9	5.60	68.0	0.17	5.0	0.85
Ave.	13.9	5.63	67.3			0.83
29	10.2	5.50	89.1	0.25	5.0	1.3
	10.6	5.40	93.2	0.37	5.0	1.9
Ave.	10.4	5.45	91.1			1.6
36	9.1	5.40	87.3	0.34	5.0	1.7
	8.0	5.20	102.0	0.56	5.0	2.8
Ave.	8.6	5.30	94.7			2.3
48	7.3	5.30	93.4	0.53	5.0	2.7
	7.8	5.20	91.1	0.56	5.0	2.8
Ave.	7.6	5.25	92.8			2.8
72	6.9	6.45	78.1	0.09	50.0	4.5
	6.9	6.40	85.7	0.16	50.0	8.0
Ave.	6.9	6.43	82.0			6.3
96	9.2	7.50	49.6	0.84	250.0	214.0
	7.4	7.40	54.2	0.23	250.0	57.5
Ave.	8.3	7.45	51.9			135.8

FIGURE 3

THE RELATIONSHIP OF GROWTH, pH AND RIBOFLAVIN CONTENT OF CULTURES
OF *A. GOSSEYII* GROWN IN A PEPTONE MEDIUM AND THE DRY WEIGHTS OF
THEIR EXTRACTED CELLS

There is no indication of riboflavin synthesis until after the first 24 hours. The synthesis proceeds slowly until at 72 hours there is a very rapid increase in the production of the vitamin. As Figure 3 shows, riboflavin is not synthesized in appreciable amounts until several hours after the maximum growth has been reached. The data leaves little doubt that riboflavin synthesis occurs after maximum growth in a glucose-peptone medium.

The changes in pH during growth were the same as those described by Tanner, et al., (100). During the first 36 to 40 hours of growth, there is a gradual decrease in pH to a value of approximately 5.3. The pH shows a rapid increase after 45 hours reaching a value of pH 7.45 at 96 hours. It is to be noted that the pH increase closely parallels the increase in riboflavin synthesis. It has not been determined whether the pH increase is a result of riboflavin synthesis or riboflavin synthesis is a result of the changes causing the pH increase. It is possible in the peptone medium that the concentration of some intermediate related to riboflavin synthesis must reach a critical value before the vitamin production can begin and that the cells must be of a certain age or stage of growth before this limiting intermediate can be made available to the riboflavin synthesizing mechanisms of the cells.

2. The Fractionation of Peptone on a Cellulose Column. A sample of Difco-peptone was fractionated on a Whatman powdered cellulose column, in order to determine which amino acid or peptide components were associated with the formation of riboflavin by A. gossypii. The column was prepared according to the procedure previously given. The eluate was collected in tubes, manually, every 3 to 4 ml. until a total of 40 separate

volumes had been collected. The liquid in each tube was chromatographed with the circular chromatography technique as it was collected from the column. R_f values of components were calculated and tubes with components having the same R_f value were combined. The R_f values and the tubes that were combined are shown in Table 4.

TABLE 4
THE R_f VALUES OF CHROMATOGRAPHICALLY
SEPARATED FRACTIONS OF PEPTONE

Fraction Number	Tubes Combined	Average R_f Values of the Rings
I	1-7	0.78
II	8-10	0.75, 0.57
III	11-13	0.72, 0.57, 0.41
IV	15,16,18	0.58, 0.41, 0.30
V	19-30	0.36, 0.27
VI	31-34	Approx. 0.27
VII	35-40	Very faint ring

The fractions were concentrated to dryness over CaCl_2 in a vacuum desiccator at room temperature. The R_f values of the fractions indicated that peptone contained at least four distinct components.

3. The Growth and Riboflavin Production of *A. gossypii* with Peptone Fractions as Nitrogen Sources. The peptone fractions obtained in the previous experiment were used as nitrogen sources for studying the growth and the formation of riboflavin by *A. gossypii*. The standard basal

medium with 0.05 per cent instead of 0.1 per cent NaCl was prepared as previously described and the different peptone fractions were added to the basal medium to give individual media which were sterilized by autoclaving. The sterile media were inoculated with a washed cell suspension of A. gossypii and placed on the shaker in the constant temperature room. A control with peptone as the nitrogen source was run in the same way. After 96 hours, the flasks were removed from the shaker and examined visually for growth and riboflavin production (the formation of yellow pigment). The growth and pigmentation were estimated on the basis of an arbitrary maximum value of +4 for the peptone control. The lower values were in proportion to the control value, with the designation $\pm$ indicating a value slightly less than the corresponding + value. The results obtained are shown in Table 5.

TABLE 5

THE GROWTH AND PIGMENTATION OF ASHBAYA GOSSYPII ON MEDIA CONTAINING PEPTONE FRACTIONS AS NITROGEN SOURCES

Fraction Number	Growth	Pigmentation
I	+ 4	$\pm$ 1
II	+ 3	0
III	0	0
IV	+ 4	$\pm$ 1
V	+ 4	0
VI	+ 4	+ 3
VII	+ 2	0
Peptone Control	+ 4	+ 4

The growth compared favorably with the control in all fractions except III and VII. Fraction VI was the only fraction which showed any appreciable pigmentation, however, there was evidence of slight pigmentation in fractions I and IV. It appears that fraction VI contains the component or components which account for the stimulation of riboflavin synthesis when peptone is used as a nitrogen source. It is to be noted that this fraction contained the low R_f value ninhydrin positive components which would indicate the presence of basic amino acids and/or peptides, under the chromatographic conditions employed.

4. The Proximate Qualitative Chromatographic Analysis of the Amino Acids in the Peptone Fractions. In order to obtain some indication of the amino acids present in the peptone fractions, portions of the fractions were hydrolyzed with 6 N HCl in sealed glass tubes by autoclaving at 15 pounds steam pressure for 30 minutes. Portions of the hydrolyzates were chromatographed by the circular technique and the R_f values calculated for the resulting rings. These R_f values were compared with those for the known amino acids in Table 2 to find the amino acids that were possibly present. No effort was made to identify the individual amino acids. The results are presented in Table 6. The R_f values of these rings may vary slightly from the values of the pure amino acids since interactions among the amino acids and their hydrochloride salts will affect the migration; however, relative locations of the rings can be compared with the known locations of the pure amino acids.

The chromatogram of fraction VI, the fraction which allowed appreciable pigmentation, showed that the concentration in the three rings

TABLE 6

THE R_f VALUES OF THE RINGS AND POSSIBLE AMINO ACIDS IN THE
HYDROLYZATES OF PEPTONE FRACTIONS

Peptone Fraction	R_f Value																
I	0.66	Valine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.58	Tyrosine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.34	Threonine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.26	Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.19	Phenylalanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
II	0.73	Methionine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.60	Lysine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.32	Leucine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.25	Isoleucine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
III	0.66	Histidine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.60	Glycine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.52	Glutamic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.33	Cystine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.25	Aspartic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.17	Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IV	0.71	Alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.65		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.34		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.27		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.19		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(Table continued on following page)

of lower R_f value was much greater than the comparable rings of any of the other fractions. These rings indicate the possible presence of the dicarboxylic and basic amino acids, which would indicate a relationship between these amino acids and riboflavin formation in A. gossypii. However, compounds other than amino acids could be present in this fraction and exert some effect on the synthesis of the vitamin.

5. The Effect of Various Amino Acid Combinations on the Growth and Riboflavin Formation of A. gossypii. The previous experiment presented some evidence that certain amino acids were concentrated in the fraction of peptone which gave appreciable pigmentation with A. gossypii. These amino acids, in various combinations, were used as nitrogen sources in a series of media in order to determine if combinations of pure amino acids could stimulate the growth and pigmentation obtained on peptone. The basal medium was prepared with 0.05 per cent NaCl and the amino acids were added in various concentrations to give a constant 40 mg. of nitrogen per 100 ml. of each medium. The nitrogen was determined on the basis of L-amino acids present in each medium. After the addition of the amino acids, the pH was adjusted to 6.5 - 6.8 and the media sterilized by autoclaving. The amino acid composition of the media are given in Table 7. Since arginine seemed to be present in each peptone fraction it was arbitrarily chosen as the initial amino acid of the series which was tested.

The sterile media were inoculated with a 36 hour washed cell suspension of A. gossypii and incubated on the shaker in the constant temperature room. The media were examined in the same manner as in the previous experiment at various time intervals to estimate the growth and

TABLE 7

THE AMINO ACID COMPOSITION OF SYNTHETIC MEDIA

Medium No.	Amino Acids* (g/100 ml)									
	L-arg	L-leu	DL-pal	DL-ala	L-glut	DL-thr	glyc	DL-aspt	L-hist	DL-meth
1	0.160									
2	0.120	0.100								
3	0.120	0.050	0.100							
4	0.120	0.050	0.050	0.026						
5	0.080	0.050	0.050	0.026	0.100					
6	0.080	0.025	0.050	0.026	0.100	0.042				
7	0.080	0.025	0.025	0.026	0.100	0.042	0.010			
8	0.060	0.025	0.025	0.026	0.100	0.042	0.020	0.047		
9	0.040	0.025	0.025	0.026	0.100	0.042	0.020	0.047	0.025	
10	0.040	0.025	0.025	0.026	0.075	0.042	0.020	0.047	0.025	0.046

* L-arginine HCl, L-arg; L-leucine, L-leu; DL-phenylalanine, DL-pal; DL-alanine, DL-ala; L-glutamic acid, L-glut; DL-threonine, DL-thr; glycine, glyc; DL-aspartic acid, DL-aspt; L-histidine HCl, L-hist; DL-methionine, DL-meth.

pigmentation. The results are presented in Table 8.

TABLE 8

THE EFFECT OF AMINO ACID COMBINATIONS ON GROWTH
AND PIGMENTATION OF ASHBYA GOSSYPII

Medium No.	Time							
	24 hours		48 hours		72 hours		96 hours	
	Growth	Pigment	Growth	Pigment	Growth	Pigment	Growth	Pigment
1	sl	0	± 1	0	+ 2	0	+ 3	0
2	+ 1	0	+ 2	sl	+ 2	sl	+ 4	+ 1
3	sl	0	sl	0	+ 1	0	+ 2	0
4	sl	0	+ 1	0	+ 1	0	+ 3	sl
5	+ 1	0	+ 2	0	+ 3	0	+ 4	0
6	+ 2	0	+ 3	0	+ 4	0	+ 4	sl
7	+ 2	0	+ 3	0	+ 4	0	+ 4	sl
8	+ 2	0	+ 3	0	+ 4	sl	+ 4	sl
9	+ 3	sl	+ 4	+ 1	+ 4	+ 2	+ 4	+ 3
10	+ 3	0	+ 4	0	+ 4	0	+ 5	0

At the end of the first 24 hours, medium number 6 showed an increased growth over the previous media, indicating that L-glutamic acid is probably associated with the growth of the organism. A similar increased growth was noted with medium number 9 when L-histidine HCl was added. Medium number 9 also gave evidence of slight pigmentation which may relate histidine to riboflavin formation. After 48 hours, growth had increased in all the media except number 3 which may have been inhibited by DL-phenylalanine since the presence of D-amino acids does sometimes

exert such effects. At 48 hours, pigmentation continued in medium number 9 and a slight coloration appeared in medium number 2 which contained only arginine and leucine as nitrogen sources. The final examination of the media at 96 hours indicated good growth in all cases except medium number 3. There was slight pigmentation in media numbers 4, 6, 7 and 8; moderate pigmentation in medium number 2 and appreciable pigmentation in medium number 9.

The final results seem to indicate interaction effects of the amino acids which are present in the media. These effects could be the results of interfering concentrations or merely the presence of the amino acids themselves. When L-leucine was added to L-arginine HCl, pigmentation resulted which would seem to relate leucine to riboflavin formation but the addition of DL-phenylalanine to the leucine-arginine medium prevented the pigment formation which should have been expected to continue. An even greater effect on riboflavin synthesis was shown by the addition of methionine which completely reversed the effect of histidine and inhibited the riboflavin formation which had been demonstrated in medium number 9. It is evident that interactions must be taken into consideration whenever combinations of amino acids are used for studying riboflavin synthesis in A. gossypii.

6. Further Studies of the Effects of Amino Acids on the Growth and Biosynthesis of Riboflavin by A. gossypii. In the preceeding experiment it was noted that the addition of histidine to the other amino acids resulted in an increased formation of riboflavin. This experiment was designed to study the effect of histidine further and to determine which amino acid relationships were directly concerned with the formation of

riboflavin by A. gossypii. The standard basal medium with 0.05 per cent NaCl was used throughout the experiment and was designated as "basal". Since histidine seemed to be a stimulator for riboflavin synthesis, it was chosen as the base amino acid in the first part of the experiment and was added to the basal as the hydrochloride in a concentration of 0.012 g. or an equivalent of 2.24 mg. nitrogen, per 100 ml. of medium to make the base medium for studying the effects of other added amino acids. This base medium was distributed into flasks, into each of which except one was added singly the other amino acids used. Thus one flask contained histidine as the nitrogen source, whereas the other flasks contained histidine plus another amino acid. Control flasks were also set up for peptone as the nitrogen source and the basal alone without a nitrogen source. The media were adjusted to pH 6.5 with NaOH and sterilized. The sterile media were inoculated with a 36 hour washed cell suspension and incubated on the shaker for 4 days at 28° C. After incubation the growth was estimated visually in the same manner as the previous experiment. The riboflavin was estimated visually by examining the flasks in a beam of ultraviolet light and estimating the amount of fluorescence as compared with that from peptone as + 4. This allowed the detection of a much smaller concentration of pigment than could be determined by color. The composition of the media and the effects on growth and riboflavin formation are shown in Table 9.

Glutamic acid was the only amino acid which showed a substantial stimulation of the growth of A. gossypii, however, the addition of leucine, alanine, threonine or asparagine did increase the growth slightly over the histidine alone. Only leucine and threonine exhibited any stimulating

TABLE 9

THE EFFECT OF ADDED AMINO ACIDS ON THE GROWTH AND RIBOFLAVIN FORMATION BY ASHBYA GOSSYPII IN A HISTIDINE BASE MEDIUM

Medium No.	Base Medium	Medium Composition		Growth	Fluorescence (riboflavin)
		Nitrogen Source Added	Concentration (g/100 ml)		
1	Basal	L-histidine HCl	0.012	+sl	0
2	Med. #1	L-glutamic acid	0.21	+ h	0
3	Med. #1	L-leucine	0.19	+ 1	sl
4	Med. #1	L-arginine HCl	0.08	sl	0
5	Med. #1	Glycine	0.11	sl	0
6	Med. #1	DL-aspartic acid	0.39	sl	0
7	Med. #1	DL-alanine	0.26	+ 1	0
8	Med. #1	DL-threonine	0.34	+ 1	sl
9	Med. #1	DL-serine	0.30	sl	0
10	Med. #1	Asparagine	0.09	+ 1	0
11	Basal	Peptone	0.14	+ h	+ h
12	Basal	None	----	0	0

effect on pigment formation; however, the effect was small. At the concentration used, it is evident that histidine cannot serve as the sole nitrogen source for growth and riboflavin formation by A. gossypii.

Since these results indicated that good growth was obtained with L-glutamic acid and L-histidine HCl as nitrogen sources, the next part of this experiment was performed using these amino acids as the principal nitrogen sources. L-glutamic acid and L-histidine HCl were added to the

basal to give 20.0 mg. and 2.4 mg., respectively, of L-amino acid nitrogen per 100 ml. of medium to make the base medium for studying the effects of other added amino acids in this part of the experiment. This base medium was distributed into flasks, into each of which except one was added singly the other amino acids used. Thus one flask contained glutamic acid and histidine as the nitrogen sources, whereas the other flasks contained glutamic acid, histidine and another amino acid. The media were adjusted to pH 6.5 with NaOH, sterilized, inoculated with a 36 hour washed cell suspension and incubated on the shaker for 4 days at 28° C. The composition of the media, and the results determined in the same way as in the first part of the experiment are presented in Table 10.

The addition of L-histidine HCl to the glutamic acid medium resulted in decreased growth as compared to the glutamic acid medium. There was also decreased growth when DL-serine or glycine was added to the glutamic acid-histidine medium, whereas the addition of L-arginine HCl increased the growth noticeably. Riboflavin formation occurred to a slight extent when L-leucine or DL-threonine was added to the glutamic acid-histidine medium, however, the addition of DL-alanine or glycine resulted in a moderate formation of the vitamin. The effects of alanine or glycine are of interest, since neither of these amino acids has previously shown any effect on riboflavin formation when added to the histidine medium in the first part of the experiment.

In the last part of this experiment, a base medium was prepared by adding L-glutamic acid, L-histidine HCl and L-arginine HCl to the basal for the nitrogen sources, since this combination of amino acids had given the best growth without riboflavin formation above. This base medium was

TABLE 10

THE EFFECT OF ADDED AMINO ACIDS ON GROWTH AND RIBOFLAVIN SYNTHESIS
IN A L-GLUTAMIC ACID - HISTIDINE HCl BASE MEDIUM

Medium No.	Base Medium	Medium Composition		Growth	Fluorescence (riboflavin)
		Nitrogen Source Added	Concentration (g/100 ml)		
1	Basal	L-glutamic acid	0.210	+ 3	0
2	Med. #1	L-histidine HCl	0.012	+ 2	0
3	Med. #2	L-leucine	0.025	+ 3	sl
4	Med. #2	DL-threonine	0.040	+ 3	sl
5	Med. #2	DL-serine	0.032	± 2	0
6	Med. #2	L-arginine HCl	0.010	+ 4	0
7	Med. #2	DL-isoleucine	0.050	+ 1	0
8	Med. #2	DL-alanine	0.035	+ 3	+ 1
9	Med. #2	Glycine	0.015	± 2	+ 1
10	Med. #2	L-lysine HCl	0.016	+ 3	0
11	Basal	Peptone	0.155	+ 4	+ 4
12	Basal	None	-----	0	0

used to study the effects of other added amino acids and was distributed into flasks, into each of which except one was added singly the other amino acids used. This gave one flask which contained glutamic acid, histidine and arginine as the nitrogen sources, whereas the other flasks contained glutamic acid, histidine and arginine plus another amino acid. A peptone control flask was set up also. The media were adjusted to pH 6.5 with NaOH, sterilized, inoculated with a 36 hour washed cell

suspension and incubated on the shaker for 4 days at 28° C. The composition of the media, and the results determined in the same way as in the first part of the experiment are presented in Table 11.

TABLE 11

THE EFFECT OF ADDED AMINO ACIDS ON GROWTH AND RIBOFLAVIN SYNTHESIS
IN A GLUTAMIC ACID - HISTIDINE - ARGININE BASE MEDIUM

Medium No.	Base Medium	Medium Composition		Growth	Fluorescence (riboflavin)
		Nitrogen Source Added	Concentration (g/100 ml)		
1	Basal	L-glutamic acid L-histidine HCl L-arginine HCl	0.210 0.012 0.010	+ 3	0
2	Med. #1	L-leucine	0.025	+ 2	+ 3
3	Med. #1	DL-threonine	0.040	+ 3	s1
4	Med. #1	DL-alanine	0.035	+ 3	s1
5	Med. #1	Glycine	0.015	+ 3	s1
6	Basal	Peptone	0.172	+ 4	+ 3

The growth of *A. gossypii* was good in all media except medium number 2 where the addition of L-leucine decreased the growth slightly. However, the addition of L-leucine increased the riboflavin formation sharply, approximately equaling that of the peptone control. The addition of DL-threonine, DL-alanine or glycine to medium number 1 gave only a slight formation of riboflavin.

This experiment has again served to point out the interaction effects of amino acids on the growth and riboflavin synthesis by Ashby

gossypii. The results seem to indicate that riboflavin synthesis is not just the result of one or two amino acids in the medium but rather the result of the combined action of several amino acids and other factors. L-glutamic acid, alone or in combination with L-arginine HCl and small concentrations of L-histidine HCl, has been shown to adequately serve as a nitrogen source for the growth of A. gossypii, however, it appears to have little direct effect on the synthesis of riboflavin. L-leucine showed some relationship to riboflavin synthesis as indicated by the increased riboflavin formation when it was added to the glutamic acid-histidine-arginine medium; however, a decreased growth was noted in this medium when appreciable riboflavin was formed. The results obtained with DL-threonine, DL-alanine and glycine indicate some relationship to riboflavin formation but the effect of these amino acids may be masked by the presence of the other amino acids in the medium. If one can assume that threonine, alanine and glycine are related to riboflavin formation, their lack of greater effect may be due to limited production of an essential link in the riboflavin synthesizing system, this link not being limiting when leucine was the amino acid added.

7. The Effect of Serial Transfers of Cells of *A. gossypii* on Synthetic Media, on Growth and Riboflavin Formation. Since the inocula for the different experiments were prepared from cells grown on yeast extract-peptone agar stock slants, there was a possibility that some substance affecting riboflavin formation was being carried over in the inoculum even though the cells were washed two times with sterile distilled water. In order to test this possibility, it was decided to see if such substances would be diluted by serial transfer on two types of synthetic

media, media that support growth but not riboflavin formation and media that support both.

Several solid media were prepared from the basal with 0.05 per cent NaCl, by adding different nitrogen sources including peptone as a control to give the compositions shown in Table 12.

TABLE 12

THE COMPOSITION OF MEDIA FOR STUDYING THE EFFECT OF SERIAL TRANSFER OF CELLS ON GROWTH AND RIBOFLAVIN FORMATION

Medium No.	Base Medium	Nitrogen Source Added	Concentration Nitrogen Source (g/100 ml)
1	Basal	Peptone	0.178
2	Basal	L-glutamic acid L-arginine HCl	0.210 0.010
3	Med. #2	L-leucine	0.025
4	Med. #3	L-histidine HCl	0.012

The media were made solid by adding 1.8 per cent Difco-agar and were slanted after sterilization. Two slants of each media were inoculated with cells of A. gossypii taken directly from a 48 hour yeast extract-peptone agar slant without washing and were incubated at 28° C. in the incubator room. After four days, cells on these slants were transferred to fresh slants of the same medium composition and were incubated. This procedure was repeated after another 4 days, making transfers to fresh slants giving a total of three transfers including the original transfer from the yeast extract-peptone agar slant. The growth

was examined visually and the riboflavin formation was estimated by observing the yellow fluorescence of the vitamin under ultraviolet light on all slants after being incubated 6 or 7 days. The results of the study are shown in Table 13.

TABLE 13

THE EFFECT OF SERIAL TRANSFER OF CELLS OF *ASHBYA GOSSYPII* ON GROWTH AND RIBOFLAVIN FORMATION ON PEPTONE AND SYNTHETIC MEDIA

Medium No.	First Transfer*		Second Transfer**		Third Transfer*	
	Growth	Fluorescence	Growth	Fluorescence	Growth	Fluorescence
1	+	h	+	h	+	h
2	+	h	+	h	+	h
3	+	h	+	h	+	h
4	+	h	+	h	+	h

* Growth and fluorescence after 6 days incubation.

** Growth and fluorescence after 7 days incubation.

The results suggest that there may be some carry over in the original transfer to the peptone medium since there appeared to be a decreased pigmentation in the first and second transfers. In the other media there was little indication of a carry over effect. In media numbers 3 and 4 the pigmentation was noticeably scattered throughout the growth on the slant rather than being generally distributed as the pigmentation on the peptone slants. This may indicate a localization of riboflavin formation on these synthetic media as a result of local deposition of some flavogenic substance carried over from the preceeding medium when the cells were streaked on the slants; however, this spotty

pigmentation was approximately equal in distribution for all transfers. There was no pigmentation with any transfers on medium number 2; however, the growth was equal to that of the other media.

To investigate further the possible carry over of substances that might have effects on growth or riboflavin formation, a similar test was made using liquid media instead of the solid media. The procedure was the same as that for the solid media except the agar was omitted and incubations were carried out on the shaker. In this case there was little difference noted among the first, second and third transfers in peptone flasks, both with respect to growth and pigmentation. The other media showed good growth; however, in contrast to the slants, there was no evidence of riboflavin formation.

Since the first test indicated that there was a possibility of a carry over, another test was carried out on a series of solid synthetic media containing different amino acids. The amino acids, L-glutamic acid and L-arginine HCl, in concentrations of 0.210 g. and 0.010 g., respectively, per 100 ml. of medium, were added to the basal with 0.05 per cent NaCl to give a completely synthetic medium which would support good growth of A. gossypii; however, it would not allow pigmentation. For purposes of clarity and brevity this glutamic acid-arginine medium will be designated "GA". The GA medium served as the base medium and was distributed into flasks into each of which except one was added other amino acids singly and at a concentration of 0.015 g. per 100 ml. of base medium. Thus one flask, as a control, contained the GA medium and the other flasks contained the GA medium plus single amino acids. The media were made into solid form by adding 1.8 per cent agar and the sterilized agar was slanted.

The original inoculation of the media was made from a 48 hour old yeast extract-peptone stock agar slant and subsequent transfers of the growth on the special media were every 48 hours onto fresh special media of the same composition. A total of three transfers, including the original, were made. The growth and pigmentation were examined as previously described except that a + 5 value was arbitrarily chosen as the maximum growth value. The composition of the media and the results are recorded in Table 14.

In general, the results indicated that there was decreased growth in the second and third transfers as compared with the original transfer. The riboflavin formation followed a similar pattern, occurring approximately 24 hours later than the appearance in the original transfer on similar media. Since the media in this experiment could not be considered optimum for the growth of the organism, it would be expected that the second transfer after the original would be slower in starting growth since the necessary conditions for a rapid initial cell growth may not be available. The appearance of the growth on the synthetic media slants was also very much different than the growth on the peptone medium slants. On the synthetic media the growth was very often tough and wrinkled with the mycelium growing tenaciously to the agar, making it very difficult to transfer the cells to fresh slants. In contrast, the growth on a peptone slant was very soft and spongy, making it easy to remove cells from a slant with an inoculation loop for transfer. These results show that there was apparently a carry over of some substance or substances that had a slight effect on growth and riboflavin formation, as was shown by the difference between the first and second transfers.

TABLE 14

THE EFFECT OF SERIAL TRANSFER ON THE GROWTH AND PIGMENTATION
OF A. GOSSYPII ON THE GLUTAMIC ACID - ARGININE
BASE MEDIUM WITH ADDED AMINO ACIDS

Amino Acids*** Added to GA	First Transfer						Second Transfer						Third Transfer					
	18 hrs.			72 hrs.			120 hrs.			148 hrs.			72 hrs.			96 hrs.		
	G	P	%	G	P	%	G	P	%	G	P	%	G	P	%	G	P	%
Glycine	+4	±1	5	+5	+1	5	+2	0	+4	±1	0	+4	+1	0	+5	+2	0	+3
DL-serine	+2	0	+4	0	+5	±1	±1	+2	0	±1	0	+4	±1	0	+5	±1	+1	0
DL-threonine	+2	0	+3	0	+4	±1	±1	+2	0	±1	0	+4	±1	0	+4	±1	0	+3
DL-alanine	+4	0	+4	0	+5	+2	+2	+2	0	+3	0	+4	±1	0	+4	±1	+2	0
DL-isoleucine	+2	0	+4	0	+5	±1	±1	+1	0	+2	0	+3	0	+3	±1	±1	+1	0
DL-phenylalanine	+3	0	+4	0	+5	±1	±1	+2	0	+4	±1	±1	±1	0	+5	±1	+2	0
L-tyrosine	+3	0	+5	±1	±1	±1	±1	+2	0	±1	±1	±1	±1	0	+5	±1	+2	0
DL-tryptophane	+3	0	+4	0	+5	±1	±1	+2	0	+3	0	+4	±1	0	+5	±1	+2	0
Asparagine	+3	0	+4	0	+5	±1	±1	+2	0	+3	0	+4	±1	0	+5	±1	+2	0
L-cystine	+2	0	+4	±1	±1	±1	±1	+2	0	+3	0	+4	±1	0	+5	±1	+2	0
DL-methionine	+2	0	+4	0	+5	±1	±1	+2	0	+3	0	+4	±1	0	+5	±1	+2	0
L-lysine HCl	+4	±1	+5	+1	±1	±1	±1	+2	0	+4	±1	±1	±1	0	+5	±1	+2	0
DL-valine	+2	0	+4	0	+5	±1	±1	+2	0	+3	0	+4	±1	0	+5	±1	+2	0
L-proline	+2	0	+4	±1	±1	±1	±1	+2	0	+3	0	+4	±1	0	+5	±1	+2	0
L-leucine	+3	0	+5	±1	±1	±1	±1	+2	0	+3	0	+4	±1	0	+5	±1	+2	0
Control**	+3	0	+4	0	+5	0	0	+2	0	+3	0	+3	0	0	+5	0	+2	0

* G is Growth; P is Pigmentation.

** GA medium alone.

*** Amino acids added in concentration of 0.015 g. per 100 ml. GA medium.

Since the growths and riboflavin formations were about the same for the second and third transfers, it seems that the substances were reduced in concentration by transfer.

On the agar slants, all of the amino acids, except valine, showed some stimulation of riboflavin formation when they were added to the GA medium; however, there were variations in degree of effect on riboflavin formation. Of the amino acids used, glycine, DL-alanine, L-tyrosine, DL-tryptophane, L-cystine, L-lycine HCl, L-proline and L-leucine exhibited the greatest stimulation of riboflavin synthesis.

In addition to the test just described, a study was made of the effect on riboflavin synthesis of serial transfers of A. gossypii cells in liquid GA medium. The glutamic acid-arginine medium was prepared from the basal medium with 0.05 per cent NaCl as previously described for the preparation of solid media except the 1.8 per cent Difco-agar was omitted. The washed cells from a 48 hour culture of A. gossypii, grown on a yeast extract-peptone stock agar slant, were inoculated into the GA medium and shaken for 48 hours at 28° C. After this 48 hour period, the cells from the GA medium were removed, centrifuged, washed and resuspended as the inoculum for the second transfer to fresh GA medium which was again shaken for 48 hours. At the end of the second 48 hour period of incubation the growth from the second transfer of cells was removed, centrifuged, washed and resuspended as the new inoculum. These cells were inoculated into a peptone medium in which the basal had been supplemented with 0.5 per cent Difco-peptone as the nitrogen source. At the same time that these serially transferred cells were inoculated into the peptone medium, a peptone control was inoculated with washed cells from a yeast extract-peptone slant.

The flasks were incubated for 5 days on the shaker at 28° C. with a set of five flasks used for each type of inoculum. The turbidimetric growth and fluorometric riboflavin determinations were made on the flasks and the results obtained are shown in Table 15.

TABLE 15

THE GROWTH AND RIBOFLAVIN FORMATION OF *A. GOSSYPII* ON A PEPTONE MEDIUM INOCULATED WITH SERIAL TRANSFER CELLS

Sample Number	Control Cells		Serial Transfer Cells	
	Turbidity (% trans.)	Riboflavin (ug/ml)	Turbidity (% trans.)	Riboflavin (ug/ml)
1	8.4	22.1	8.3	30.0
2	8.2	48.3	8.5	16.9
3	8.2	31.8	9.5	22.5
4	8.9	18.7	8.9	16.9
5	<u>Contaminated</u>		<u>8.4</u>	<u>20.8</u>
Average	8.4	30.2	8.6	21.4

The data in Table 15 show that the average growth was approximately the same in either case. The average yield of riboflavin was approximately 29 per cent greater in the peptone medium inoculated with the yeast extract-peptone slant cells than in the peptone medium inoculated with the serial transfer cells. The highest individual yield was also obtained with the yeast extract-peptone grown cells. It is to be noted, however, that although there was a large variation in the amounts of riboflavin found in the samples from either source of inoculum, the variation was less in the medium inoculated with the serial transfer cells. These data seem to

indicate that some factor or factors may be carried over by the yeast extract-peptone agar grown cells; however, the effect would be expected to be constant throughout a particular experiment.

8. The Growth and Riboflavin Formation of *A. gossypii* as Affected by Inocula Prepared from Cells Subjected to Different Numbers of Washings. The studies on serial transfers of inocula indicated the possibility of a carry over effect on riboflavin synthesis from cells grown on the yeast extract-peptone agar slants. If this factor were loosely bound to the surface of the cells, it might be expected that differences in cells, obtained after a different number of washings, might be demonstrated. This experiment was performed to see if such an effect could be demonstrated.

The medium for this experiment was prepared from the GA medium to which was added 0.015 g. of glycine and 1.8 g. of Difco-agar per 100 ml. of the medium. The test cells were taken from a 48 hour stock slant of *A. gossypii* grown on yeast extract-peptone agar. Some of the cells from this stock slant were transferred to duplicate slants of the test medium. The rest of the cells were washed with 10 ml. of distilled water, centrifuged and resuspended in distilled water. The washings were repeated to obtain a total of four washings. Transfers of cells after each washing were made to duplicate slants of the test medium. All of the slants were incubated at 28° C. for 45, 72 or 102 hours. After incubations, cultures were examined for growth and riboflavin formation in the same manner as in the previous experiment. The results are given in Table 16.

The number of washings had no effect on growth or riboflavin formation. This would seem to indicate that any factor being carried over

TABLE 16

THE GROWTH AND PIGMENTATION ON THE GLUTAMIC ACID - ARGININE - GLYCINE (GAGly) MEDIUM INOCULATED WITH CELLS WASHED A DIFFERENT NUMBER OF TIMES

Number of Washings	Period of Incubation					
	45 hrs.		72 hrs.		102 hrs.	
	Growth	Pigment	Growth	Pigment	Growth	Pigment
0	+ 3	0	+ 4	± 1	+ 5	+ 2
0	+ 3	0	+ 4	0	+ 5	+ 2
1	+ 3	0	+ 4	0	+ 5	+ 2
1	+ 3	0	+ 4	± 1	+ 5	+ 2
2	+ 3	0	+ 4	0	+ 5	+ 2
2	+ 3	0	+ 4	± 1	+ 5	+ 2
3	+ 3	0	+ 4	0	+ 5	+ 2
3	+ 3	0	+ 4	0	+ 5	+ 2
4	+ 3	0	+ 4	0	+ 5	+ 2
4	+ 3	0	+ 4	0	+ 5	+ 2

over by the cells from the yeast extract-peptone agar slants must be either bound relatively tightly to the cells or contained within the cells.

9. The Effect of Miscellaneous Nitrogen Sources on Growth and Riboflavin Formation. Several different nitrogen sources were tested to ascertain their effects on the growth and riboflavin formation by A. gossypii. A medium was prepared for each nitrogen source by adding 0.5 g. of the single nitrogen source to 100 ml. of the basal, the nitrogen sources being NH_4NO_3 , KNO_3 , $(\text{NH}_4)_2\text{SO}_4$, ammonium acetate, ammonium citrate and urea. The results in all the media showed no growth or riboflavin formation, indicating that A. gossypii probably cannot grow or produce riboflavin on simple nitrogen sources but must require more complex organic nitrogen

sources.

Although the inorganic nitrogen sources did not support growth or riboflavin formation when used as the only nitrogen source in a medium, it was possible that they might have some effect when added to a medium containing organic nitrogen sources. In order to test this possibility, a medium was prepared by adding 0.5 g. of peptone and 0.5 g. of NH_4NO_3 to 100 ml. of the basal. The medium was sterilized and inoculated in the usual manner and incubated on the shaker at 28°C . After 4 days of incubation the flasks showed a good growth of A. gossypii but very little riboflavin formation, as apparent from the lack of pigmentation. It was evident that the addition of NH_4NO_3 to the medium in the presence of peptone resulted in a decreased synthesis of riboflavin, since the peptone alone had been shown previously to support growth and riboflavin formation.

This effect was studied further with KNO_3 and ammonium acetate. A peptone medium was prepared by adding 0.5 g. of peptone to 100 ml. of the basal and the resulting medium was used to prepare two other media, one containing 0.5 g. of KNO_3 per 100 ml. of the medium and the other containing 0.5 g. of ammonium acetate per 100 ml. of the medium. The media were inoculated, sterilized and incubated in the usual manner and examined visually for growth and pigmentation. The growth in both media was very good; however, in the medium containing the KNO_3 there was no appreciable pigmentation, whereas in the medium containing the ammonium acetate there was a normal yellow pigmentation present. From these results it appeared that the nitrate ion, at the concentration used, might act in some way to inhibit the formation of riboflavin when the ion was

in the presence of peptone. This fact may be of use in further studies of the riboflavin metabolism of A. gossypii.

10. The Treatment of Peptone with Permutit. It has been shown previously in Experiment number 3 that the more basic components of peptone seemed to be concerned with riboflavin formation in A. gossypii, rather than the other components. If these basic components could be removed from the peptone in some simple manner, then their relationship to riboflavin synthesis could be further demonstrated. Since Permutit has been used to remove ammonia in urine analysis, it might also remove the more basic components of peptone.

The experiment was carried out on a solution containing 0.5 g. of peptone dissolved in 50 ml. of distilled water. Five grams of washed Permutit was added to the solution which was stirred for several minutes. The Permutit was removed by filtration and the filtrate containing the unabsorbed parts of peptone was made into a medium by adding the other component parts of the basal to the filtrate and diluting it to 100 ml. An untreated peptone medium was prepared by adding 0.5 per cent of peptone to the standard basal. The Permutit treated and untreated media were inoculated with a washed 48 hour cell suspension of A. gossypii and incubated for 4 days at 28° C. on the shaker. The growth was then measured by obtaining the dry weight of an aliquot of cells before extraction of riboflavin and the riboflavin was measured fluorometrically in the usual manner. The results of the experiment are shown in Table 17.

The data show a great contrast between the treated and the untreated peptone media. The growth in the treated peptone medium was

TABLE 17

THE GROWTH AND RIBOFLAVIN FORMATION ON MEDIA CONTAINING UNTREATED
AND PERMUTIT TREATED PEPTONE

Medium	Dry Weight Cells (mg/20 ml)				Riboflavin (ug/ml)			
	1	2	3	Ave.	1	2	3	Ave.
Untreated Peptone	78.0	79.8	92.0	83.2	47.5	67.5	110.8	75.3
Permutit Treated Peptone	120.0	110.4	118.2	116.2	4.8	5.1	5.3	5.1

now approximately 25 per cent greater than that in the untreated peptone medium which indicated that Permutit did not remove anything from the peptone which was essential for the growth of the organism. In fact, the treatment of the peptone may have removed something that was inhibitory to the growth of the organism.

The effect on riboflavin formation was even more pronounced than the effect on growth. The average yield of riboflavin was 5.1 ug. per ml. for the treated peptone medium and 75.3 ug. per ml. for the untreated peptone medium or fifteen times as much riboflavin formed in the untreated over the treated media. When Permutit was treated with a 1 M solution of KOH, the absorbed substances from the extraction were freed into the solution. The eluate, from the Permutit, used in the peptone treatment, showed the presence of ninhydrin positive substances when spotted on filter paper and sprayed with the ninhydrin developing reagent. This verified that something was removed from the peptone when treated with

Permutit and the factors removed may be concerned in the synthesis of riboflavin. A circular chromatogram of the eluate from the Permutit showed that only one wide ring was present and this heavy ring had an R_f value of 0.30 - 0.31 which corresponds with one of the R_f values of the components in the riboflavin stimulating combined fraction found in the column separation of peptone.

A portion of the Permutit eluate was hydrolyzed with 6 N HCl in a sealed glass tube by autoclaving the tube at 15 pounds steam pressure for 1 hour. A sample of the eluate and hydrolyzed eluate were chromatographed by the two dimensional techniques on Whatman number 1 sheet filter paper with the n-butanol-acetic acid-water and pyridine-tertiary amyl alcohol-water solvents. The chromatogram of the unhydrolyzed eluate showed one large ninhydrin positive spot which had R_f values of approximately 0.13 and 0.21, respectively, with the two solvents. The chromatogram of the hydrolyzed eluate showed nine distinct spots. Their R_f values were calculated and the possible amino acids identified by comparison of the values with those for pure amino acids. The R_f values and corresponding possible amino acids are given in Table 18.

To study this absorbed fraction further, a medium was prepared from the regular GA medium by adding 70 mg. of L-lysine HCl, 20 mg. of L-histidine HCl, 15 mg. of glycine, 40 mg. of DL-threonine, 30 mg. of L-proline, 10 mg. of L-leucine and 5 mg. of phenylalanine to 100 ml. of the medium. These amino acids were selected from those in the above table because in previous experiments all of them gave some indication of being related to riboflavin synthesis. The medium was adjusted to pH 6.5 with a solution of KOH, sterilized and inoculated with a 48 hour washed

TABLE 18

THE R_f VALUES AND POSSIBLE AMINO ACIDS PRESENT IN A CHROMATOGRAPHED,
HYDROLYZED PERMUTIT EXTRACTED FRACTION OF PEPTONE

Butanol Solvent R_f	Pyridine Solvent R_f	Possible Amino Acid Composition
0.16	0.17	Lysine Arginine
0.17	0.29	Histidine
0.20	0.39	Serine
0.26	0.19	Aspartic acid Glutamic acid
0.24	0.27	glycine
0.30	0.38	threonine
0.35	0.42	proline
0.61	0.59	valine
0.72	0.68	leucine isoleucine phenylalanine

cell suspension. After incubation on the shaker for 4 days at 28° C., the flasks were examined visually for growth and riboflavin formation. This medium gave excellent growth of the organism but did not show any appreciable riboflavin formation. These results indicate the strong effect due to interrelationships of amino acids since under other conditions these amino acids showed an effect on riboflavin synthesis; however, when they were together in these concentrations there was no appreciable effect on the formation of the vitamin. It appears that further study is necessary

in order to determine an amino acid medium which will give good yields of riboflavin.

11. The Growth and Riboflavin Synthesis on the Glutamic Acid-Arginine Medium with Different Concentrations of Glycine. Since glycine was one of the amino acids which stimulated the formation of riboflavin in the GA medium, a series of GA media were prepared to determine the effect of different concentrations of glycine on growth and riboflavin formation when the glycine was added to the GA medium. The GA media were prepared in the same way as that in Experiment number 7 except different amounts of glycine were also included in the media. The GA medium without added glycine served as a control for the experiment. The media were sterilized and duplicate flasks inoculated with a 48 hour washed cell suspension of A. gossypii. After incubation for 4 days on the shaker at 28° C., the growth was determined turbidimetrically and the riboflavin was determined fluorometrically as in previous experiments. The concentrations of glycine in the media and the results of the experiment are reported in Table 19.

The addition of different concentrations of glycine had very little apparent effect on growth, as shown by the turbidity readings for the different flasks, even though there was a considerable increase in the total nitrogen in the media, as compared with the GA control medium.

The addition of glycine to the GA medium resulted in an increased formation of riboflavin. The increase in riboflavin was rather gradual and reached a maximum value of 15.2 ug. per ml. when 30.0 mg. of glycine was added to 100 ml. of the GA medium. When 40.0 mg. or 50.0 mg. of glycine was added to the GA medium, there was no further increase in riboflavin

TABLE 19

THE GROWTH AND RIBOFLAVIN SYNTHESIS OF A. GOSSYPII
IN GLUTAMIC ACID - ARGININE (GA) MEDIA
WITH ADDED AMOUNTS OF GLYCINE

Glycine Added to GA Medium (mg/100 ml)	Turbidity (% Transmittance)			Riboflavin (ug/ml)		
	1	2	Ave.	1	2	Ave.
0	9.7	9.2	9.5	0.61	0.59	0.60
1.0	9.9	10.2	10.0	1.1	1.1	1.1
2.5	10.2	8.5	9.3	3.4	3.4	3.4
5.0	11.2	14.2	12.6	3.8	3.8	3.8
10.0	10.1	11.2	10.6	2.8	2.8	2.8
15.0	10.9	12.1	11.4	7.5	7.5	7.5
20.0	10.0	9.2	9.6	11.2	10.3	10.8
30.0	10.0	10.8	10.4	15.3	15.0	15.2
40.0	10.0	13.8	11.9	10.6	10.0	10.3
50.0	11.0	12.7	11.8	12.5	12.5	12.5

formation which might indicate that a limiting condition had been reached and, as a result, further increases of other substances might be necessary for further conversion of glycine to the intermediate in riboflavin synthesis.

These results indicate a relationship of glycine to riboflavin formation in the GA medium; however, it appears that other substances must be necessary for further riboflavin synthesis in this medium. This relationship of glycine to riboflavin formation in A. gossypii was in agreement with the results of Plaut (67) who showed that tagged glycine

was incorporated into the ring portion of the riboflavin molecule.

12. The Effect of Different Concentrations of Peptone on Growth and Riboflavin Formation in a Glutamic Acid - Arginine - Glycine Medium.

The preceding experiment showed that glycine stimulated riboflavin formation in the GA medium but the yield was considerably less than that which could be obtained from peptone at approximately the same nitrogen concentration. If peptone contained some factor which was responsible for the formation of riboflavin, the addition of peptone to the glutamic acid-arginine-glycine (GAGly) medium should show an increased formation of riboflavin. To test this, the GAGly medium was prepared by adding 20 mg. of glycine to 100 ml. of the regular GA medium which has been described previously. The peptone was added to the GAGly medium in amounts ranging from 0.001 mg. to 100 mg. per 100 ml. of the medium, duplicate flasks being set up for each amount of peptone added, as well as, a duplicate set of flasks containing the GAGly medium as a control. The media were sterilized in the usual manner and inoculated with a 48 hour washed cell suspension of A. gossypii. The inoculated flasks were incubated on the shaker for 4 days at 28° C. After incubation, the flasks were removed and the resulting growth and riboflavin were measured using the dry weight of cells and fluorometric analysis, respectively. The concentrations of the peptone added and the results obtained are presented in Table 20.

The addition of increasing amounts of peptone to the GAGly medium resulted in a gradual increase in the growth of the organism, except for the two lower amounts of added peptone where the growth was erratic. The addition of 100 mg. of peptone to the GAGly medium resulted in the largest increase in growth, amounting to approximately two times that of the GAGly

TABLE 20

THE GROWTH AND RIBOFLAVIN SYNTHESIS IN A GLUTAMIC ACID -
 ARGININE - GLYCINE (GAGly) MEDIUM CONTAINING
 VARIOUS CONCENTRATIONS OF PEPTONE

Concentration of Peptone (mg/100 ml)	Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)			(ug/mg dry cells)
	1	2	Ave.	1	2	Ave.	
0*	53.6	53.8	53.7	16.0	---**	16.0	6.0
0.001	84.2	----***	84.2	7.5	---***	7.5	1.8
0.01	36.0	32.8	34.4	8.0	9.8	8.9	5.2
0.10	53.2	----***	53.2	10.8	---***	10.8	4.1
1.00	63.0	74.6	68.8	16.0	8.6	12.3	3.5
5.00	85.2	73.8	79.5	5.7	5.7	5.7	1.4
25.00	97.0	----***	97.0	5.7	---***	5.7	1.2
50.00	77.4	85.4	81.4	5.7	2.8	4.3	1.0
100.00	97.8	104.4	101.1	2.2	2.4	2.3	0.5

* GAGly control.

** Lost because of breakage.

*** Contaminated Flask.

control medium.

The addition of different concentrations of peptone to the GAGly control medium did demonstrate a marked effect on riboflavin formation. The GAGly control showed the formation of 16.0 ug. of riboflavin per ml. which was the maximum produced in this experiment. The addition of peptone to the GAGly medium resulted, in general, in a decreased formation for riboflavin, with the lowest value of 2.3 ug. per ml. being obtained

in the medium to which 100 mg. of peptone had been added per 100 ml. of medium. The addition of the 100 mg. of peptone reduced the riboflavin formed to about one-eighth the value of the control. There appears to be an inverse relationship between growth and riboflavin formation, since the increased growth was accompanied by the decreased formation of riboflavin. This is especially evident as shown by the amount of riboflavin produced per mg. dry weight of cells which decreased as the amount of peptone was increased. The addition of the peptone to the GAGly medium might have increased the growth metabolism of the organism, therefore, decreasing the availability of those intermediates necessary for riboflavin formation. The results of this experiment serve to reiterate the importance of the interactions of nitrogen sources in the formation of riboflavin by A. gossypii.

13. The Effect of the Components of the Glutamic Acid - Arginine "

Glycine Medium on the Synthesis of Riboflavin in a Peptone Medium. The unexpected inhibition of riboflavin formation by the addition of peptone to the GAGly medium raised the question as to which part of the synthetic medium might be causing the effect, if this were the reason for the effect. This experiment was performed in an effort to obtain some information concerning the effects of the different components of the GAGly medium on the riboflavin formation in a peptone medium.

A peptone medium was prepared by adding 100 mg. of peptone to 100 ml. of the basal, which was used for the preparation of other media. There were three media used for this experiment and their composition was as follows: Medium number 1, the control, was the peptone medium prepared as just described, Medium number 2 was the peptone medium to which 0.21 g.

of glutamic acid and 0.01 g. of arginine HCl had been added per 100 ml. of medium and Medium number 3 was the peptone medium supplemented with 0.02 g. of glycine per 100 ml. of medium. Duplicate flasks of the media were sterilized and inoculated with a 36 hour washed cell suspension of *A. gossypii*. The inoculated media were incubated for 5 days on the shaker at 28° C. The growth was examined visually and the riboflavin determined fluorometrically in the usual manner. The results of the experiment are given in Table 21.

TABLE 21

THE SYNTHESIS OF RIBOFLAVIN IN A PEPTONE MEDIUM CONTAINING COMPONENTS OF THE GLUTAMIC ACID - ARGININE - GLYCINE (GAGly) MEDIUM

Amino Acids Added to Peptone Medium (g/100 ml)	Riboflavin (ug/ml)		
	1	2	Ave.
0	10.3	9.4	9.9
L-glutamic acid (0.21) L-arginine HCl (0.01)	0.8	0.8	0.8
Glycine (0.02)	13.1	10.6	11.9

Since growth in all the media was very good, the possibility of any inhibition effects due to the addition of components of the GAGly medium was eliminated. The riboflavin formation averaged 9.9 ug. per ml. in the peptone medium which was a reasonable value for the amount of nitrogen present in the medium. The addition of the glutamic acid-arginine components of the GAGly medium to the peptone medium caused the riboflavin formation to be reduced to an average of 0.8 ug. per ml.,

whereas, the addition of glycine alone produced a slight increase in riboflavin formation as compared to that formed in the peptone medium. These results indicated that the glutamic acid-arginine portion of the GAGly medium must have caused the inhibition of riboflavin formation by A. gossypii in the peptone medium and that this was probably the effect observed in Table 20 of the preceeding experiment. Van Lanen, et al., (105) reported that dicarboxylic acids and their derivatives interfered with the synthesis of riboflavin by A. gossypii. Under the condition of growth when peptone and glutamic acid are both present in the same medium, glutamic acid may be converted to α -ketoglutaric acid through deamination, and thereby giving a sufficient concentration of the dicarboxylic acid to exert an inhibitory effect on the formation of riboflavin.

14. The Effect of a Cell Extract of A. gossypii on Growth and Riboflavin Formation. Since the experiments on serial transfer (Experiments 13, 14 and 15) had indicated the possibility of a factor being carried over with the yeast extract-peptone agar slant cells, an extraction of this type cell was made. The extract was prepared from pigments cells of A. gossypii which were removed with an inoculating loop from several 48 to 72 hour yeast extract-peptone agar slants. The cells were placed in a mortar and ground in a small amount of distilled water with a mixture of ground glass and sand, in order to break up the cells. The ground mixture was centrifuged and the centrifugate, about 10 ml., removed as the cells extract which was stored in the freezing compartment of a refrigerator.

Two media were prepared with the following compositions: Medium number 1, designated GA, contained 0.210 g. of glutamic acid and 0.010 g.

of arginine HCl per 100 ml. of the basal and Medium number 2, designated GAGly, contained 0.020 g. of glycine per 100 ml. of the GA medium. Before sterilization of the GA and GAGly media, 0.1 ml. of the A. gossypii cell extract was added to each 20 ml. of the different media in Erlenmeyer flasks. After sterilization, duplicate flasks of the media were inoculated with a 36 hour washed cell suspension of A. gossypii and incubated on the shaker for 4 days at 28° C.

At the completion of the incubation period growth was observed and riboflavin determined fluorometrically. Growth was very good in all but one flask which showed no growth at all, this flask being one containing the GA medium plus the cell extract. The results of the fluorometric determinations of riboflavin are shown in Table 22.

TABLE 22

THE SYNTHESIS OF RIBOFLAVIN BY A. GOSSYPII IN GLUTAMIC ACID - ARGININE (GA) AND GLUTAMIC ACID - ARGININE - GLYCINE (GAGly) MEDIA CONTAINING 0.1 MILLILITER OF CELL EXTRACT

Medium	Riboflavin (ug/ml)		
	1	2	Ave.
GA	2.7	No growth	2.7
GAGly	21.8	26.9	24.3

The addition of the cell extract to the GA medium resulted in a riboflavin yield which was only slightly greater than that which has been reported for the GA medium in other experiments. This increased yield may have been due to the small amount of riboflavin added with the 0.1 ml.

of the cell extract which did contain a small amount of the vitamin. The effect of the cell extract was very evident in the GAGly medium where a riboflavin yield of 24.3 ug. per ml. of medium was reported. This was approximately double that which was normally produced in this medium without the presence of added extract and therefore indicated the presence of some factor in the extract which stimulated the formation of riboflavin. In observations of the progress of growth in this experiment, it was noted that a perceptible amount of pigmentation had formed in Medium number 2 after only 12 hours of incubation which was several hours sooner than previously observed. Since the cell extract was added to the medium before sterilization, the stimulation factor must be some heat stable compound or compounds, contained in the cell extract or resulting from the autoclaving of the cell extract, which accounts for the stimulation of riboflavin formation in the presence of glycine. Autoclaving would also eliminate the probability that an enzyme or enzyme system in the cell extract was stimulating the synthesis of the vitamin. The relationship of glycine to riboflavin synthesis was more firmly established as a result of these findings since the increased riboflavin formation was noted only in the GAGly medium.

15. The Effect of Chromatographic Fractions of an *A. gossypii*

Cell Extract on Riboflavin Formation. The previous experiment had shown that the extract of *A. gossypii* cells stimulated the formation of riboflavin in the GA medium with added glycine. In an effort to determine if the whole extract or only part of the extract was responsible for the increased riboflavin formation, the extract was chromatographed on filter paper strips and fractions were tested.

The extract was chromatographed on strips of Whatman number 1 filter paper with the n-butanol-acetic acid-water solvent mixture as the resolving system. After 0.05 ml. of the cell extract had been applied to each of three filter paper strips and dried, the strips were placed in the solvent containers and chromatographed for approximately 6 hours. At this time, the strips were removed from the solvent and allowed to air dry. Two of the strips were sprayed with indicator reagents and the third was scanned with ultraviolet light. Strip number 1 was sprayed with a ninhydrin solution to test for the presence of ninhydrin positive components, such as, amino acids, peptides and some proteins. Strip number 2 was developed with a phenol red indicator solution which would locate some of the basic components since the indicator turns red at pH values over 8.5. Strip number 3 was examined under ultraviolet light to locate any fluorescent spots, especially riboflavin which gives a strong yellow fluorescence. Strip number 3 was also used in the preparation of special media for determining the effects of the substances (on riboflavin formation) located at the different spots as shown by the developing agents on strips 1, 2 and 3. The results of the chromatographic examination of the strips, together with the fractions to be incorporated into the media, are shown in Table 23.

The GA and GAGly media were prepared from the basal as described in the preceeding experiment. No cell extract was added to the GA medium or one flask of the GAGly medium; however, 0.05 ml. of the whole extract was added to one flask of the GAGly medium, thus giving three controls for the experiment. Since only one chromatographic strip was used in this experiment, only one flask was used for each fraction to be tested. The

TABLE 23

THE CHROMATOGRAPHIC COMPOSITION OF AN *A. GOSSYPII* CELL EXTRACT
FROM A BUTANOL - ACETIC ACID - WATER SOLVENT SEPARATION

Fraction Number	Developing Agent	R _f Values	Color Formed
1 (origin)	-----	0	None
2	Ninhydrin	0.23	Light orange
3	Ninhydrin	0.31	Purple
5	Ninhydrin	0.58	Pink
6	Ninhydrin	0.75	Light pink
3	Phenol Red	0.28	Red
3	UV light	0.30	Blue fluor.
4	UV light	0.45*	Yellow fluor.
7** (top)	-----	----	-----

* Riboflavin.

** Portion of strip from last spot to solvent front.

chromatographic fractions, numbered according to Table 23, were cut from strip number 3 and placed into separate flasks of the GAGly medium. These media, together with the GA and GAGly controls, were sterilized and the sterile media inoculated with a 44 hour old washed cell suspension of *A. gossypii*. After an incubation period of 108 hours on the shaker at 28° C., the fluorometric determination of the riboflavin was made. The results are shown in Table 24.

Since filter paper had been introduced into the flasks with the chromatographic fractions, the dry weight of cells was not determined,

TABLE 24

THE EFFECT OF CHROMATOGRAPHIC CELL EXTRACT FRACTIONS ON RIBOFLAVIN
SYNTHESIS BY A. GOSYPII IN A GLUTAMIC ACID -
ARGININE - GLYCINE (GAGly) MEDIUM

Medium Number	Principal Medium	Added Extract Fraction	Riboflavin (ug/ml)
1	GA*	---	0.6
2	GAGly*	---	4.3
3	GAGly plus 0.05 ml. extract*	---	19.4
4	GAGly	1	16.9
5	GAGly	2	5.3
6	GAGly	3	3.8
7	GAGly	4	2.5
8	GAGly	5	4.7
9	GAGly	6	2.8
10	GAGly	7	4.1

* Control media.

however, observations of growth indicated that it was excellent in all of the media. The routine daily examination of the progress of the experiment showed that pigmentation had appeared in Media 3 and 4 after 36 hours of incubation. As would be expected, the riboflavin formed in the GA medium was rather small, while that formed in the GAGly medium, although less than had previously been reported, was noticeably greater than the GA medium control. The stimulation of riboflavin formation was again apparent when only 0.05 ml. of the whole cell extract was added to the

GAGly medium before sterilization. An examination of the results when the chromatographic fractions were added to the GAGly medium showed that Fraction number 1 stimulated riboflavin formation to approximately the same extent as the whole cell extract. This was unexpected since it was felt that the stimulation would probably come from ninhydrin positive portions of the cell extract. Since only 0.05 ml. of the cell extract was applied to the origin of the paper strip, the amount of substances remaining at the point of application after the finish of the chromatogram must have been very small because the density of the ninhydrin color developed on the spots would indicate a rather heavy concentration of substances, especially at the R_f values of 0.31 and 0.58. Fractions numbered 4 and 6 gave some slight indication of being inhibitory to riboflavin formation in the GAGly medium. The remainder of the fractions seemed to show little effect on riboflavin synthesis. Fraction number 1 may have contained some of the larger molecular cell components, such as, proteins, nucleotides, etc., which would show little, if any, migration in the n-butanol-acetic acid-water solvent system.

16. A Further Study of the Effect of Cell Extracts of *A. gossypii* on Growth and Riboflavin Formation. The data presented in the two preceding experiments, showed that the extract of *A. gossypii* cells from yeast extract-peptone agar slants stimulated the formation of riboflavin in the presence of glycine. Whether this same stimulatory effect could be shown with extracts from shaker cells grown on the yeast extract-peptone medium, remained to be demonstrated. A large flask was set up as a fermenter in order to grow a large number of *A. gossypii* under aerated conditions. Three liters of medium were prepared by adding 1.5 g. of

Difco-peptone, 1.5 g. of Difco-yeast extract and 6.0 g. of glucose to 3 liters of distilled water. The sterilized medium was inoculated with a 48 hour old cell suspension of A. gossypii and incubated at room temperature with continuous aeration by a stream of sterile diffused air.

After 84 hours of growth, a portion of the medium was removed and the cells filtered off to give 18.4 g. of wet cells. These cells were ground in a small amount of distilled water with sand and powdered glass to disrupt the cells and the resulting extract of the cells was obtained by centrifugation. This extract will be referred to as the 84 hour aqueous cell extract.

The remainder of the medium was removed after 132 hours and the cells removed by filtration to yield 58.8 g. of wet cells. A 15 g. portion of the cells was dried with cold acetone to yield 2.8 g. of acetone dried cells and an acetone extract of the cells. The remaining 43.8 g. of cells was extracted in the same manner as the 84 hour cells to give a 132 hour aqueous cell extract. The aqueous extracts of the different age cells were made in order to determine at which phase of growth the stimulatory factor or factors were most predominate. All extracts were stored in the refrigerator when not in use. A 20 ml. portion of the 132 hour aqueous cell extract was lyophilized to give 0.17 g. of dried solids or 8.5 mg. per ml. of extract. The GA and GAGly media for testing the extracts were prepared from the basal according to previous procedures. The other media for the experiment were prepared by the addition of the cell extracts and dried cells, to the GAGly medium. After the addition of the cell extracts and dried cells, the media were sterilized, inoculated with a 24 hour old washed cell suspension of A. gossypii

and incubated on the shaker for 4 days at 28° C. The results of the dry weight of cells and the fluorometric riboflavin determinations are shown in Table 25. In addition, the riboflavin content of each of the added extracts was determined and the average yields corrected for the riboflavin added initially, although much of the riboflavin would have been destroyed in autoclaving the media at a near neutral pH.

TABLE 25

THE GROWTH AND RIBOFLAVIN FORMATION IN A GLUTAMIC ACID - ARGININE -
GLYCINE MEDIUM WITH ADDED CELL
EXTRACTS AND ACETONE DRIED CELLS

Medium Used	Component Added/20 ml.	Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)		
		1	2	Ave.	1	2	Ave.
GA**	---	46.4	91.6	69.0	1.1	3.7	2.4
GAGly**	---	50.4	72.8	61.6	10.7	16.2	13.5
GAGly	0.1 ml. of 84 hr. extract	66.4	63.6	65.0	16.5	10.0	13.1 12.5*
GAGly	0.1 ml. of 132 hr extract	50.8	44.8	47.8	38.1	44.3	41.2 39.2*
GAGly	0.1 ml. acetone extract	44.2	35.4	39.8	5.5	10.5	8.0 6.4*
GAGly	1.0 mg. acetone dried cells	76.5	62.4	69.0	5.5	11.7	8.5

* Corrected averages for riboflavin present in the extracts.

** Control media

The results of this experiment verified the previous findings concerning the stimulation of riboflavin synthesis by aqueous extracts of A. gossypii cells and showed that cells grown on a liquid yeast extract-peptone

medium could produce the riboflavin stimulating factor. The data showed that the stimulation factor was present only in the 132 hour aqueous cell extract which may indicate that it was formed in the older cells after appreciable riboflavin had been produced. This does not eliminate the possibility that it could be in the 84 hour aqueous cell extract but if there, the concentration must be less than that which could be demonstrated under the conditions employed. The acetone cell extract and the acetone dried cells apparently did not contain the stimulation factor but rather appeared to contain factors which were inhibitory to the synthesis of riboflavin in the GAGly medium. Although the 132 hour aqueous cell extract increased the formation of riboflavin, it produced a decreased growth of the organism, again showing the inverse relationship between growth and riboflavin formation.

17. The Colorimetric Analysis of Riboflavin. Since the fluorometric method required a considerable amount of time, it seemed desirable to make an effort to develop a colorimetric assay method to reduce the time necessary to make an analysis of the riboflavin produced in a set of experimental media. On this basis some of the accuracy of the fluorometric determinations could be sacrificed in order to gain speed through the colorimetric determination. A colorimetric method would be especially suited to these studies since the riboflavin produced by cells in liquid media was comparatively free from interfering substances. The use of a colorimetric method for the assay of riboflavin had been reported by Yaw but no details were given for the procedure. To test the colorimetric method a standard curve was prepared, different size aliquots of a ground cell extract were assayed and the recoveries of riboflavin added to

aliquots were checked.

An Evelyn colorimeter, which was fitted with a 420 millimicron filter, was standardized at 100 per cent transmittance with a distilled water - dithionite solution which contained approximately 30 mg. sodium dithionite per 10 ml. of distilled water. An air blank was then recorded and the instrument adjusted to this blank throughout the remainder of any test determinations. The riboflavin samples or standard solutions of 11 to 12 ml. volume were added to calibrated colorimeter tubes and the tubes inserted into the colorimeter. The per cent transmission of the sample was read to the nearest 0.25 per cent transmittance unit and recorded as the sample reading. After the sample was read, approximately 30 mg. of granular sodium dithionite was added to the tube, the solution mixed thoroughly and the mixed solution allowed to stand for 30 to 60 seconds. While one tube was standing, another tube in the assay was read and treated in a similar manner, thus no time was lost in waiting for reduction of the riboflavin. After reduction, the dithionite treated sample was also read on the galvanometer scale to the nearest 0.25 per cent transmittance unit and recorded as the correction reading. The sample and correction readings were then converted to absorbance values and the correction subtracted from the sample to give the corrected absorbance value produced by the riboflavin in the sample. The riboflavin in the sample was determined from a standard curve which related the absorbance value to known concentrations of riboflavin. All riboflavin determinations that were accomplished colorimetrically in succeeding experiments followed the above procedure. The data in Table 26 was obtained from a colorimetric analysis of a series of standard

riboflavin solutions by the above procedure. The data are plotted as a standard curve in Figure 4.

TABLE 26

ABSORBANCE VALUES FOR KNOWN RIBOFLAVIN SOLUTIONS

Riboflavin (ug/ml)	Per Cent Transmittance		Absorbance		
	Sample	Correction	Sample	Correction	Corrected Value
0	-----	100	-----	-----	0
5	58.75	87.00	0.2310	0.0605	0.171
10	34.75	78.75	0.459	0.104	0.355
15	21.00	70.00	0.678	0.155	0.523
20	13.75	62.75	0.862	0.202	0.660
25	9.25	55.75	1.034	0.254	0.780

An examination of Figure 4 shows a linear relationship between absorbance and riboflavin concentrations up to 15 ug. per ml., beyond which the curve falls off slightly. However, the relationships are sufficiently linear throughout the curve to allow for a reasonable estimate of the riboflavin concentration.

In order to test this method further, the riboflavin content of a cell extract, obtained by grinding cells of A. gossypii in a small amount of distilled water with ground glass and sand, was determined using different aliquots of the cell extract. The aliquots of the cell extract were tested to check the reproducibility of the method in the presence of increasing concentrations of interfering substances which

FIGURE 4

A STANDARD CURVE FOR THE COLORIMETRIC
ANALYSIS OF RIBOFLAVIN

were introduced by the addition of the aliquots of the cell extract. Four unknown samples were prepared, by adding 0.1, 0.3, 0.5 and 1.0 ml. of the cell extract to approximately 15 ml. of distilled water, and autoclaved in the presence of sodium acetate buffer, as previously described, in order to solubilize any bound riboflavin in the cell extract. After autoclaving, the samples were diluted to 25 ml. with distilled water before being tested.

A second set of four samples was prepared as just described, except 1 ml. of a standard riboflavin solution, containing 25 ug. of riboflavin per ml., was added to each aliquot sample of the cell extract before autoclaving. This second set of samples was used to test the method for recoveries of known amounts of riboflavin which were added to the unknown samples. The results for the determinations are given in Table 27.

The data show good agreement between different aliquots of the unknown cell extract and adequate recoveries of added riboflavin. When the values for the riboflavin content of the original cell extract were averaged, a value of 34.4 ug. of riboflavin per ml. was obtained.

Twenty days prior to the colorimetric determinations on the cell extract, this same extract had been assayed fluorometrically and found to contain 40.5 ug. of riboflavin per ml. A comparison of these two values indicates the colorimetric method gave results about 14 per cent below the fluorometric method. Considering the storage period of 20 days in the refrigerator and the fact that some riboflavin was destroyed during storage, the colorimetric assay of the cell extract was in satisfactory agreement with the fluorometric determination, at least for making

TABLE 27

THE DETERMINATION OF RIBOFLAVIN IN ALIQUOTS OF AN EXTRACT OF CELLS
AND A CHECK OF RECOVERY OF ADDED RIBOFLAVIN

Extract (ml)	Riboflavin Added (ug/ml)	Transmittance (Per Cent)		Extract Absorbance		Riboflavin	
		Sample*	Correction	Sample*	Corrected Value	Sample* (ug/ml)	Ogn'l. Extract Recovered (ug/ml)
0.1	----	81.75	89.50	0.087	0.048	1.3	32.6
0.3	----	52.00	72.50	0.284	0.139	4.1	34.2
0.5	----	32.00	58.00	0.494	0.237	7.3	36.6
1.0	----	12.25	36.50	0.912	0.438	13.5	33.8
Check of Recovery of Added Riboflavin							
0.1	1.0	71.00	86.50	0.149	0.063	2.4	1.1
0.3	1.0	45.00	69.00	0.348	0.160	5.3	1.2
0.5	1.0	30.75	59.25	0.512	0.227	8.1	0.8
1.0	1.0	11.50	37.00	0.937	0.432	14.4	0.9

* Volume of extract used diluted to 25 ml.

approximate assays. The results of the assay above indicate that the colorimetric determination of riboflavin is feasible by this method, especially where approximate measurements are of interest rather than small accurate quantitative determinations. It is believed that the method could be refined to a point where it could be used as a more quantitative method of assay for riboflavin in culture media.

18. The Effect of Glycine, L-serine and DL-threonine on Growth and Riboflavin Synthesis when Added to Different Synthetic Media. Glycine, when added to the synthetic glutamic acid-arginine medium, has been shown to be effective in stimulating the formation of riboflavin by A. gossypii (Experiment number 9). The interrelationships of glycine and serine have been reported in the metabolisms of microorganisms, therefore, the substitution of L-serine for glycine might be expected to stimulate riboflavin formation also. DL-threonine was used because of its structural similarity to serine and the slight effect on riboflavin synthesis it had previously shown.

The media were prepared from the basal as follows: the glutamic acid-arginine (GA) medium, 0.210 g. of L-glutamic acid and 0.010 g. of L-arginine HCl per 100 ml. of basal, the aspartic acid-arginine (AA) medium, 0.210 g. of L-aspartic acid and 0.010 g. of L-arginine HCl per 100 ml. of basal, and the asparagine-arginine (AspA) medium, 0.210 g. of asparagine and 0.010 g. of L-arginine HCl per 100 ml. of basal. The other media were prepared by the addition of 20.0 mg. of glycine, 28.0 mg. of L-serine or 63.5 mg. of DL-threonine to each of the GA, AA and AspA media, the amino acids being added on the basis of the same L-alpha amino acid nitrogen content. The media were adjusted to pH 6.5 with NaOH and sterilized in

the usual manner. After sterilisation duplicate flasks of each media were inoculated with a 36 hour washed cell suspension and incubated for 4 days at 28° C. on the shaker. The dry weights of cells and colorimetric riboflavin determinations on the different media were made; these values are shown in Table 28.

The growth was approximately the same in the case of the GA and AA media without added amino acids but the yields of riboflavin were very small. The fact that the vitamin synthesis in the AA medium was twice that in the GA medium, may or may not be significant. However, in the AspA medium without added amino acids appreciable riboflavin was synthesized although there was decreased growth of the organism. It has been observed that there was usually less growth in a medium compared with the control, when riboflavin was produced. When glycine was added at a concentration of 20 mg. per 100 ml. to each of the three media, a noticeable increase in the riboflavin formed was observed, especially in the AA and AspA media. This would further substantiate the relationship of glycine to riboflavin synthesis with aspartic acid being probably more closely related to the synthesis than glutamic acid. The results with asparagine would seem to agree with this, since asparagine is known to be metabolically related to aspartic acid. The increase in riboflavin synthesis shown by asparagine without glycine may be due, in part at least, to the amide nitrogen which is present. Again in the GA and AA media, there was decreased growth when appreciable riboflavin was formed. The substitution of L-serine or DL-threonine for glycine failed to stimulate vitamin synthesis to the same extent as the glycine. This may indicate that serine and threonine are not as directly involved in riboflavin

TABLE 26

THE EFFECT OF GLYCINE, L-SERINE OR DL-THREONINE ON GROWTH AND RIBOFLAVIN SYNTHESIS IN GLUTAMIC ACID - ARGININE, (GA) ASPARTIC ACID - ARGININE (AA) AND ASPARAGINE - ARGININE (AspA) MEDIA

Medium	Amino Acid Added	Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)		
		1	2	Ave.	1	2	Ave.
GA	None	113.6	99.6	106.6	1.3	1.0	1.2
GA	Glycine	97.6	96.0	96.8	10.5	16.0	13.3
GA	L-serine	102.2	91.6	96.9	2.5	0.5	1.5
GA	DL-threonine	126.4	123.6	125.0	2.8	2.0	2.4
AA	None	109.6	104.8	107.2	2.8	2.0	2.4
AA	Glycine	89.8	80.2	85.0	20.2	28.8	24.5
AA	L-serine	103.8	99.2	101.5	4.8	4.3	4.6
AA	DL-threonine	136.8	83.4	110.1	4.3	2.8	3.6
AspA	None	57.8	67.0	62.4	7.8	12.2	10.0
AspA	Glycine	73.4	----*	73.4	30.5	----*	30.5
AspA	L-serine	91.2	65.6	78.4	17.5	10.5	14.0
AspA	DL-threonine	66.6	64.8	65.7	9.5	9.5	9.5

* Contaminated flask.

synthesis or it may be due to the fact that under the conditions of the experiment they were not utilized as readily as glycine. If glycine is required for riboflavin formation, the rate of its formation may be a factor; therefore, serine might not stimulate the formation of riboflavin, even though it is thought that glycine is derived from serine in many microorganisms, because the glycine might not be formed fast enough.

Serine and threonine have been reported by Goodwin and Pendlington to be related to riboflavin synthesis in E. ashbyii, whereas, glycine had no effect. The data from this experiment would indicate that the reverse may be the case with A. gossypii.

19. The Effect of L-histidine HCl on Growth and Riboflavin Formation in Glutamic Acid-Arginine and Glutamic Acid-Arginine-Glycine Media. Yaw had reported that histidine was effective in stimulating riboflavin synthesis in E. ashbyii but the addition of histidine to experimental media in previous experiments of this study failed to show any appreciable effect on riboflavin formation with A. gossypii. Since only one concentration of histidine had been used in the previous experiment, the effect of histidine might not have been apparent; therefore, tests were performed using different concentrations of histidine in the GA medium and the GA medium with glycine (GAGly) of the preceding experiment. The GAGly medium contained 20 mg. of glycine per 100 ml. of the GA medium. In the first of two tests, the inoculum was prepared from a 36 hour washed cell suspension of A. gossypii which was added to the sterile media and incubated on the shaker for $\frac{1}{2}$ days at 28° C. After incubation the dry weight of cells and colorimetric riboflavin determinations were made. These values and the amounts of histidine added are reported in Table 29.

The lower concentrations of histidine showed a stimulation of growth in both media and an inhibition on riboflavin synthesis in the GAGly medium. At higher concentrations of histidine there was a decreased growth of the organism. Histidine may have some effect on riboflavin synthesis in the GA medium especially at high concentrations where a slight stimulation of synthesis was shown on the basis of riboflavin per

unit dry weight of cells. The effect was also shown at higher concentrations of histidine in the GAGly medium. The data seem to indicate that there may be two conditions under which riboflavin was synthesized, namely, synthesis after cells had reached maximum growth and synthesis by histidine inhibited cells which show only negligible growth. With the lower histidine concentrations, there appeared to be an increased growth accompanied by riboflavin synthesis. It has been shown previously that riboflavin synthesis under conditions of rapid growth occurred after cells had reached maximum growth, that is to say, in this experiment riboflavin was synthesized by a relatively large number of cells for a relatively short period of time with the lower histidine concentrations. As the concentration of histidine was increased, inhibition of growth occurred and less riboflavin was formed by the decreased number of cells that had reached the maximum growth phase. At the higher concentration of histidine, growth was inhibited further; however, appreciable riboflavin was synthesized by a relatively small number of inhibited cells during the entire incubation period. It has been noted that pigmentation appeared earlier in the media containing the higher concentrations of histidine. At the histidine concentration of 25 mg. per 100 ml., the growth was inhibited in the GAGly medium to the extent of one-twentieth of the control but the synthesis of the riboflavin was not retarded, as might be expected. The high synthetic rate of the inhibited cells is shown by the ratio of riboflavin synthesized to the dry weight of cells.

The other test concerning the effect of histidine on growth and riboflavin formation was performed in the same way as the first test, using the same GAGly medium as just described; however, higher concentrations

of histidine were used to confirm the inhibition by histidine and to observe any other effects of the higher concentrations. Control media of GA and GAGly without histidine were also included. In this test, a 28 hour washed cell suspension was inoculated into duplicate flasks of the media and the inoculated flasks incubated for 4 days at 28° C. on the shaker. The amounts of added histidine, the dry weight of cells and the colorimetric determinations of riboflavin are shown in Table 30.

The results indicate an even stronger inhibition on growth by higher concentrations of L-histidine HCl. At concentrations of histidine greater than 30 mg. per 100 ml. of medium, there seems to be less total riboflavin synthesis but this may not be significant. A concentration of 40 mg. per ml. seems to be about the maximum concentration necessary, since 50 mg. per 100 ml. showed only a small difference in effect on growth or riboflavin formation over the 40 mg. concentration. However, there was a steady increase in the amount of riboflavin produced per mg. dry weight of cells, indicating the same high rate, if not higher, of riboflavin biosynthesis in the small number of inhibited cells. Since inhibition of growth can be shown without stopping riboflavin synthesis, this effect may provide a means of separating the growth and riboflavin synthesizing metabolisms of A. gossypii.

20. The Effect of Initial pH of the Medium on Growth and Riboflavin Formation in the Glutamic Acid-Arginine Medium and the Glutamic Acid-Arginine-Glycine-Histidine Medium. The inhibition of the growth of A. gossypii by histidine in the glutamic acid-arginine (GA) and glutamic acid-arginine-glycine (GAGly) media without accompanying inhibition of riboflavin synthesis, presented a very interesting problem since no

TABLE 30

THE EFFECT OF HISTIDINE HCl ON GROWTH AND RIBOFLAVIN FORMATION IN
THE GLUTAMIC ACID - ARGinine - GLYCINE (GAGly) MEDIUM

Medium	L-histidine HCl Added (mg/100 ml)	Dry Weight Cells (mg/20 ml)			(ug/ml)			Riboflavin (ug/20 ml)			(ug/mg cells) Ave.
		1	2	Ave.	1	2	Ave.	1	2	Ave.	
GA*	0	77.2	84.8	81.0	2.7	2.5	2.6	52			0.64
GAGly*	0	93.6	77.8	85.7	12.0	16.7	14.3	286			3.3
GAGly	20.0	59.2	79.8	69.5	13.8	15.5	14.6	292			4.2
GAGly	30.0	8.8	7.6	8.2	14.8	11.8	13.2	264			32.0
GAGly	40.0	2.4	1.0	1.7	13.8	10.8	12.3	246			145.0
GAGly	50.0	2.2	0.8	1.5	11.8	12.5	12.2	244			163.0

* Control media.

report of this inhibition effect had been noted in the literature. Since many inhibitions are a function of the pH at which they occur, the effect of different initial pH values, on the histidine inhibition effect, together with growth and riboflavin formation, were studied. The effect of pH on growth and riboflavin was studied in the GA medium; however, the histidine inhibition, as affected by pH, was studied only in the GAGlyH medium. The GA and GAGlyH media were prepared in the experiment as previously described. The glutamic acid-arginine-glycine-histidine (GAGlyH) medium, used in this experiment, was prepared by adding 25 mg. of L-histidine HCl to 100 ml. of the GAGlyH medium. The media were adjusted to the different initial pH values with a solution of NaOH and duplicate flasks of each medium were sterilized in the usual manner. After sterilization the media were inoculated with a 24 hour washed cell suspension and incubated for 4 days on the shaker at 28° C. After incubation, growth and colorimetric riboflavin determinations were made in the usual manner. These results and the initial pH values are presented in Table 31.

The growth of A. gossypii was moderately affected by different initial pH values in the GA medium; however, the changes were not large. In the GA medium, the data show a slight increased growth from pH 6.0 to a maximum growth at pH 6.4 which was followed by decreased growth to pH 6.8. The initial pH of the medium exhibited a more pronounced effect on growth in the GAGlyH medium. At the initial pH of 6.0 in the GAGlyH medium, the inhibitory effect of histidine was completely eliminated and growth of A. gossypii was considerably higher than even the maximum growth in the GA medium. This might indicate that at this initial pH the histidine was in a form which decreased its active concentration and

TABLE 31

THE EFFECT OF INITIAL pH OF MEDIA ON GROWTH AND RIBOFLAVIN FORMATION
IN THE GLUTAMIC ACID - ARGININE (GA) AND GLUTAMIC
ACID - ARGININE - GLYCINE - HISTIDINE (GAGlyH) MEDIA

Medium	Initial pH	Dry Weight Cells (mg/20 ml)			Riboflavin			
		1	2	Ave.	(ug/ml)		(ug/mg cells)	
					1	2	Ave.	Ave.
GA	6.0	53.6	46.4	50.0	1.0	0.5	0.8	0.3
GA	6.2	52.0	42.4	47.2	1.3	0.5	0.9	0.4
GA	6.4	53.6	65.2	59.4	1.3	0.8	1.0	0.4
GA	6.6	50.2	47.2	48.8	2.0	1.3	1.7	0.7
GA	6.8	40.0	42.4	41.2	3.5	3.5	3.5	1.7
GAGlyH	6.0	78.0	80.0	79.0	6.3	7.5	6.9	1.7
GAGlyH	6.2	21.2	10.6	15.9	7.8	8.5	8.2	11.7
GAGlyH	6.4	5.6	4.0	4.8	6.3	8.0	7.2	31.3
GAGlyH	6.6	10.2	10.4	10.3	16.7	15.0	15.9	30.9
GAGlyH	6.8	8.4	4.8	6.6	11.8	12.0	11.9	39.1

therefore it acted to stimulate growth as was shown in Experiment number 19. It could also indicate that glycine was more readily used in the growth metabolism of the organism at this pH. When the initial pH was raised to 6.2, the inhibition effect once again was apparent and the growth was decreased to approximately one-fifth of that obtained at the initial pH 6.0. The inhibition increased further at pH 6.4 but was slightly less at the initial pH values of 6.6 and 6.8. The data would indicate a pH dependency for the maximum inhibitory effect by histidine on the growth of A. gossypii.

The effect of the initial pH of the media on riboflavin formation was evident in both series of media. In the GA medium, the normal values for riboflavin formation, in the range of 0.8 ug. to 2.0 ug. per ml., were noted at initial pH values of 6.0 to 6.6, which included the normal initial pH range around pH 6.5 used in most of the previous experiments. At the initial pH of 6.8, the riboflavin formed in the GA medium rose to 3.5 ug. per ml. which was a rather high value for this medium without additional amino acids. It is possible that pH 6.8 was approaching the optimum pH for the synthesis of riboflavin and even on the GA medium the metabolism may shift somewhat to the synthesis of the vitamin. It should be pointed out, that the growth in the GA medium was least at the initial pH where the highest value of riboflavin was produced in the GA medium. Once again, notice is made of the formation of riboflavin at the expense of the growth of the organism. The riboflavin which was formed in the GAGlyH medium with the initial pH values of 6.0 through 6.4, was slightly below the normal values usually found in this medium; however, at the initial pH of 6.6, the riboflavin formed was approximately twice the value of yields in the GAGlyH medium at lower initial pH values. The initial pH of 6.6 seems to be the optimum for riboflavin synthesis in the GAGlyH medium which is in contrast to the pH 6.8 optimum for the GA medium.

21. The Effect of Imidazole on Growth and Riboflavin Synthesis.

Imidazole is a chemical compound which is structurally related to histidine in that it makes up the ring portion of the amino acid molecule. Because of this relationship, imidazole was used to determine whether this portion of the histidine molecule contributed to any of the inhibition

effect produced by histidine. The GA and GAGly media were prepared as previously described and served as controls for the experiment. The effect of imidazole was tested in media, prepared by adding 20.0 mg. and 30.0 mg. of imidazole, respectively, to separate 100 ml. volumes of the GAGly medium. For a comparison with histidine, two other media were prepared in the same manner as the imidazole media, except L-histidine HCl was used in the place of imidazole. All media were adjusted to pH 6.5 with NaOH and duplicate flasks of each medium were sterilized in the usual manner. The sterile media were inoculated with a 28 hour washed cell suspension and incubated for 4 days on the shaker at 28° C. The dry weight of cells and the colorimetric riboflavin values were determined. These values are shown in Table 32.

The controls were run in addition to the imidazole media in order to give a better comparison of some of the factors involved. The effect of imidazole was different than expected, for the addition of either concentration used, to the GAGly medium produced an increased growth which was approximately 20 per cent greater than the growth on the GA or the GAGly control media. It is evident that imidazole does not demonstrate any of the inhibition effect shown by histidine on A. gossypii.

A microscopic examination of the cells in the imidazole media showed that a large percentage of the cells were the "bulb forms" reported by Fridham and Raper (72) who showed the presence of riboflavin crystals within these cells. The number of this type cell in the imidazole media would approximate the number of the same type cells found in a 0.5 per cent peptone medium. In the other synthetic media, where good growth occurred, there was only a small percentage of these swollen cells

TABLE 32

A COMPARISON OF THE EFFECT OF IMIDAZOLE WITH THAT OF HISTIDINE ON GROWTH
AND RIBOFLAVIN SYNTHESIS IN THE GLUTAMIC
ACID - ARGININE - GLYCINE (GAGly) MEDIUM

Medium	Compound Added (mg/100 ml)	Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)		
		1	2	Ave.	1	2	Ave.
GA*	0	77.2	84.8	81.0	2.7	2.5	2.6
GAGly*	0	93.6	77.8	85.7	12.2	16.7	14.3
GAGly	L-histidine HCl (20.0)	59.2	79.8	69.5	13.8	15.5	14.6
GAGly	L-histidine HCl (30.0)	8.8	7.6	8.2	14.8	11.8	13.2
GAGly	Imidazole (20.0)	110.0	96.0	103.0	13.8	18.0	15.9
GAGly	Imidazole (30.0)	114.0	102.0	108.0	20.8	21.0	20.9

* Control media.

present, with the lower riboflavin yielding media having the fewer number of the cells. There were a very few of these riboflavin producing cells in the histidine inhibited media; however, those present were 5 to 10 times larger than the normal "bulb form" cells, which may account, to some extent, for the formation of reasonably large amounts of riboflavin under the inhibition conditions.

There was increased riboflavin formation in the imidazole media as compared to the other media but this was probably due to the increased number of cells present. As a result of these data, it is apparent that more than the imidazole portion of the histidine molecule is necessary

in the inhibition effect of histidine on the growth of A. gossypii.

22. The Effect of Different Concentrations of Glutamic Acid or Aspartic Acid on Growth, Riboflavin Formation and Histidine Inhibition in Synthetic Media. Some of the earlier experiments using the peptone medium indicated that riboflavin was formed principally after the growth of the organism had reached the maximum growth phase. Among other things, this could indicate that riboflavin was not formed until the nitrogenous nutrients had been nearly metabolized, thereby producing a nitrogen deficient condition in the medium. Most of the synthetic media used in these studies contained an appreciable amount of the main nitrogen source, namely glutamic acid or aspartic acid, which might prevent a physiologically suitable condition for riboflavin synthesis after maximum growth. This experiment was performed to determine if lower concentrations of the main nitrogen source would have any effects on the metabolism of A. gossypii in non-riboflavin producing media and in riboflavin producing inhibition media.

The media were prepared from the basal, containing 0.01 g. of L-arginine HCl per 100 ml., and by the addition of L-glutamic acid or L-aspartic acid in different concentrations to form the GA or AA media. Three GA media were prepared by the addition of 0.21 g., 0.10 g. or 0.05 g. of L-glutamic acid, respectively, to three different 100 ml. volumes of the basal plus arginine. Two AA media were prepared in a similar manner using 0.19 g. or 0.10 g. of L-aspartic acid. The glutamic acid-arginine-glycine-histidine (GAGlyH) and the aspartic acid-arginine-glycine-histidine (AAGlyH) media were prepared from each individual GA or AA medium by the addition of 20 mg. of glycine and 40 mg. of

L-histidine HCl to 100 ml. of each individual medium. The pH of each medium was adjusted to 6.5 with NaOH and duplicated flasks sterilized in the usual manner. The sterile media were inoculated with a 28 hour washed cell suspension and incubated for 4 days on the shaker at 28° C. After incubation, the dry weight of cells and colorimetric riboflavin values were determined. These values and the concentrations of glutamic and aspartic acids are given in Table 33.

TABLE 33

THE EFFECT OF DIFFERENT CONCENTRATIONS OF L-GLUTAMIC ACID AND L-ASPARTIC ACID ON GROWTH AND RIBOFLAVIN SYNTHESIS IN SYNTHETIC MEDIA

Medium	Concentration Main N Source (g/100 ml)	Dry Weight Cells (mg/20 ml)			Riboflavin			(ug/mg cells) Ave.
		1	2	Ave.	1	2	Ave.	
GA	Glutamic Acid							
GA	0.21	75.0	78.0	76.5	2.0	1.8	1.9	0.4
GA	0.10	35.6	40.0	38.8	1.5	0.5	1.0	0.1
GA	0.05	25.4	24.0	24.7	0.3	0.3	0.3	0.3
GAGlyH	0.21	2.2	3.4	2.8	7.5	8.5	8.0	2.6
GAGlyH	0.10	1.4	2.6	2.0	4.5	5.3	4.9	3.4
GAGlyH	0.05	1.4	1.2	1.3	9.5	7.5	8.5	7.6
AA	Aspartic Acid							
AA	0.19	73.4	83.4	78.4	2.3	1.8	2.1	0.4
AA	0.10	40.4	37.8	39.1	1.5	1.8	1.7	0.2
AAGlyH	0.19	2.2	2.8	2.5	9.0	8.5	8.8	7.2
AAGlyH	0.10	1.0	1.2	1.1	4.0	4.5	4.3	5.3

The reduction of the amount of the main nitrogen source in the media produced a decreased growth approximately equal in degree to the reduction of the nitrogen source. In the GA and AA media, a reduction of the concentrations of glutamic and aspartic acids by one-half produced approximately one-half of the growth obtained at the higher concentrations of 0.21 g. and 0.19 g., respectively, for the amino acids. A further decrease in glutamic acid concentration to 0.05 g. per 100 ml. of media did not produce as great a reduction in growth; however, a moderate reduction was evident.

A reduction in the riboflavin synthesized occurred in the GA and GAGlyH media, as well as the AA and AAGlyH media, when the concentrations of the main nitrogen sources were reduced to 0.10 g. per 100 ml. of media. At a glutamic acid concentration of 0.05 g. per 100 ml. of medium in the GAGlyH medium, the amount of riboflavin produced was approximately the same as that produced at the highest concentration of glutamic acid in the experiment. It is possible that at this low level of glutamic acid, the products of the glutamate metabolism may not have been at a high enough concentration to interfere with the formation of riboflavin, assuming the inhibitory effect of dicarboxylic acid derivatives on riboflavin formation is a causative factor as was previously reported. The stimulatory effect of aspartic acid on riboflavin synthesis in the presence of glycine, which was noted in a previous experiment, did not manifest itself under the inhibited condition of growth in the AAGlyH medium. This may indicate that the aspartic acid was not metabolized as readily under these conditions to the necessary intermediates for riboflavin synthesis. However, on the basis of the amount of riboflavin per

mg. dry weight of cells, the AA media did show a slightly greater effect on riboflavin formation than the corresponding GA media of the same relative composition.

23. Studies on Growth and Riboflavin Formation with the Addition of Glycine to Synthetic Media at Different Times During Incubation. In studies on metabolic pathways of biosynthesis for biological compounds, it often becomes necessary to resort to tracer techniques to follow the progress of a certain compound which may be thought to be related to the pathway. The development of the synthetic GA medium has increased the possibility of using such techniques to study riboflavin formation under defined conditions, especially since a compound, such as glycine, stimulates the formation of riboflavin when added to the medium. If a tagged compound were to be used, it would be helpful if it could be added to the medium after the growth of the organism had progressed for a period of time, and thus decrease the scattering of the tagged atom into other pathways which would be probably functioning more extensively during the early phases of growth than would the riboflavin synthesizing metabolism. With this idea in mind, it was decided to study the effect of adding glycine to several synthetic media at different times during incubation.

The base medium for the first test was prepared from the basal by the addition of 0.21 g. of L-glutamic acid and 0.01 g. of L-arginine HCl per 100 ml. to give the GA medium. The other media were prepared as follows: the GAGly medium, by adding 20 mg. of glycine per 100 ml. of GA medium; the GAGlyH medium, by adding 40 mg. of L-histidine HCl per 100 ml. of the GAGly medium, and the glutamic acid-arginine-histidine (GAH) medium, by adding 40 mg. of L-histidine HCl per 100 ml. of the GA medium.

All media were then sterilized, inoculated with a 28 hour washed cell suspension and incubated for 4 days on the shaker at 28° C. For the delayed action flasks of media, a sterile glycine solution, containing 4 mg. of glycine per ml. of distilled water, was added to the cultures in the GA and GAH media to give a concentration of 20 mg. of glycine per 100 ml. of media, the same concentration as that in the GAGly and GAGlyH media. After incubation, the dry weight of cells and the colorimetric riboflavin values were determined in the usual manner. These values and the times of the additions of glycine are given in Table 34.

TABLE 34

THE EFFECT OF GLYCINE ON GROWTH AND RIBOFLAVIN FORMATION DURING FOUR DAYS INCUBATION IN SYNTHETIC MEDIA WITH GLYCINE ADDED AT DIFFERENT TIMES DURING INCUBATION

Medium	Time Glycine Added (hours)	Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)		
		1	2	Ave.	1	2	Ave.
GA	0	57.4	66.8	62.1	2.5	2.0	2.3
GAGly	0	57.8	64.2	61.0	15.5	16.5	16.0
GA	24	51.4	60.0	55.7	5.3	6.0	5.7
GA	48	66.8	87.6	77.2	8.8	5.5	7.2
GAH	0	6.8	4.4	5.1	5.5	5.5	5.5
GAGlyH	0	14.1	12.8	13.5	16.3	14.3	15.3
GAH	24	8.4	12.1	10.3	5.8	8.3	7.1
GAH	48	7.2	6.2	6.7	6.0	6.8	6.4

The data show that riboflavin was synthesized in the GA flasks

to which glycine was added after 24 hours, or 48 hours' incubation, to approximately the same extent for either time of addition of the compound. The yield of riboflavin resulting from the late addition of glycine was considerably lower than that obtained for the GAGly medium in which the glycine was present from the beginning of the incubation. This could be explained if the glycine was necessary in the early stages of growth in order to make some intermediate in riboflavin synthesis which could not be formed in an effective concentration in the GA medium alone. However, on this basis the medium with glycine added at 24 hours would be expected to yield more riboflavin than the medium where the glycine was added at 48 hours, but this was not the case according to the results. The 48-hour addition of glycine to GA medium produced an increased growth which may indicate a more direct incorporation of glycine into growth when added to this medium after appreciable growth was already present.

This test of additions of glycine to the GA and GAH media after growth had been underway for a time was repeated, using more controls in order to obtain a clearer picture of the effect of added glycine at different times during incubation. In addition, longer incubation times after the addition of glycine were used. The sterile GA, GAGly, GAGlyH and GAH media and the sterile solution of glycine used for additions of glycine to the GA and GAH media during growth were prepared as described above. The sterile media were inoculated with a 28-hour-washed-cell suspension and incubated on the shaker at 28° C. After the addition of glycine to the incubated GA and GAH media at 24 and 48 hours, the growth was allowed to continue until a full 96 hour incubation period had been attained after each addition, which meant that there were 120 hours

and 11½ hours total incubation periods, respectively, for the flasks with glycine added during growth. Some of the flasks of GA and GAH media to which glycine had been added were removed after 96 hours total incubation in order to give short period incubations to compare with those media where glycine was added initially. After incubation, the dry cell weight and the colorimetric riboflavin values were determined in the usual manner. These values and the times of the additions of glycine are reported in Table 35.

The delayed addition of glycine to the GA medium showed approximately the same yield of riboflavin for either the 24 hour or the 48 hour addition of the glycine. The riboflavin produced in these media was less than that produced in the GAGly medium incubated for 96 hours even though the media with delayed addition of glycine did have the same incubation time with glycine in the media. This may indicate that glycine, which was added after the growth had progressed, entered into the growth metabolism of the organism rather than into the synthesis of riboflavin since the growth in the 96 hour GA medium after delayed addition of glycine was greater than that of the full 11½ hour incubation of the GAGly medium. Some of the inhibition of the synthesis of riboflavin in the GA medium with delayed addition of glycine may have been due to the formation of some riboflavin inhibitory metabolic products during the early growth of the organism.

In the histidine inhibited GAH medium, there was a slight increase in riboflavin over the GA control which may indicate a relationship of histidine to riboflavin synthesis; however, due to the inhibition of growth, the metabolism of glutamic acid and arginine may be forced into

TABLE 35

THE EFFECT OF DELAYED ADDITION OF GLYCINE ON GROWTH AND
RIBOFLAVIN FORMATION IN SYNTHETIC MEDIA

Medium	Hour Glycine Added	Incubation Time (hours)	Dry Weight Cells (mg./20 ml)			Riboflavin (ug/ml)		
			1	2	Ave.	1	2	Ave.
GA	--	96	54.3	36.0	44.7	2.5	3.2	2.9
GA	--	144	31.4	33.8	32.6	2.5	3.2	2.9
GAGly	0	48	29.6	27.4	28.5	2.8	3.0	2.9
GAGly	0	72	34.2	34.2	34.2	8.3	9.0	8.7
GAGly	0	96	34.0	39.8	36.9	7.5	10.3	8.7
GAGly	0	144	37.6	42.6	40.1	8.0	12.5	10.3
GA	24	72	35.4	33.2	34.3	5.8	5.5	5.6
GA	24	120	49.0	24.2	36.6	6.0	4.0	5.0
GA	48	48	35.4	37.2	36.3	4.8	5.3	5.1
GA	48	144	43.6	54.8	49.2	5.3	5.8	5.6
GAH	--	96	3.4	3.6	3.5	5.3	3.5	4.4
GAH	--	144	2.8	4.8	3.8	5.3	5.8	5.6
GAGlyH	0	48	7.2	6.4	6.8	3.4	3.5	3.4
GAGlyH	0	72	5.0	14.0	14.5	12.5	11.4	11.4
GAGlyH	0	96	4.4	4.2	4.3	7.5	9.3	8.4
GAGlyH	0	144	3.8	3.8	3.8	10.0	11.3	10.7
GAH	24	72	4.8	4.2	4.5	9.3	9.5	9.4
GAH	24	120	5.6	2.6	4.1	8.3	8.5	8.4
GAH	48	48	3.2	6.0	4.6	6.5	8.0	7.3
GAH	48	144	8.2	7.4	7.8	7.8	11.0	9.4

the direction of riboflavin formation. In the GAH media where glycine was added at 24 and 48 hours, the riboflavin formed was approximately equal to that produced in the GAGlyH medium where glycine was present at the time of inoculation. This would indicate that the late addition of glycine to the histidine inhibited media had little effect on the extent of riboflavin synthesis.

The small yields of riboflavin obtained after the delayed addition of glycine to the GA medium would make difficult the use of tagged compounds in studies on the biosynthesis of riboflavin under these conditions unless yields could be increased; however, their use in the histidine inhibited media might have possibilities.

24. Studies on Growth and Riboflavin Synthesis in the Glutamic Acid-Arginine-Histidine Medium with Different Concentrations of Amino Acids. The discovery of the inhibition effect of histidine offered an opportunity to study the effects of amino acids on riboflavin synthesis by eliminating, to a large extent, the growth metabolism of A. gossypii while riboflavin synthesis continued. Under these conditions, the addition of precursors or other compounds may be studied with the hope that a clearer pattern of the compounds which are related to the synthesis of riboflavin would appear. The following series of tests was such an attempt, in which a number of amino acids were studied for their effect on riboflavin formation in a histidine inhibited cell system. GA medium was prepared from the basal and contained 0.05 g. of L-glutamic acid and 0.01 g. of L-arginine HCl per 100 ml. of the basal. The lower concentration of glutamic acid as compared with 0.21 g. per 100 ml. in previous media was used to eliminate possible inhibitory effects on

riboflavin synthesis that might result from the higher concentration usually used. The lower concentration of glutamic acid was shown in a previous experiment to be satisfactory for the formation of riboflavin in the histidine inhibition medium. The GAH medium was prepared from the GA medium by adding 40 mg. of L-histidine HCl to 100 ml. of the GA medium just described. The GA and GAH media and the GAH medium plus 20 mg. of glycine per 100 ml. were used as controls throughout the experiment. Other special media except that with methionine were prepared from the GAH medium by the addition of 5.0, 10.0, 20.0 and 40.0 mg. quantities of the individual amino acids to be studied and were sterilized. The methionine, which was used in one part of the experiment, was prepared as a solution and sterilized by Seitz filtration before the addition to the sterile media. A 28 hour washed cell suspension of A. gossypii was used for each inoculation throughout the experiment. Because of space limitations on the shaker, the experiment was carried out with three amino acids and controls for each test. The inoculated media were shaken for 4 days at 28° C., after which they were removed from the shaker and the dry weight of cells and colorimetric determination of riboflavin made in the usual manner. The results along with the amino acids used, with their concentrations, are presented in Table 36.

In all of the experiments, the riboflavin produced in the GA medium controls was of a low level as had been expected. The growth in the GA medium controls was within reasonable agreement among the different experiments, ranging from 22.9 mg. to 36.6 mg. dry weight of cells per 20 ml. of medium. The GAH control gave the expected inhibition of growth in all of the experiments with the dry weight of cells ranging from less

TABLE 36

THE GROWTH AND RIBOFLAVIN FORMATION IN THE GLUTAMIC ACID - ARGININE - HISTIDINE (GAH) MEDIUM WITH DIFFERENT CONCENTRATIONS OF DIFFERENT AMINO ACIDS

Medium	Amino Acid Added (mg/100 ml)	Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)			
		1	2	Ave.	1	2	Ave.	
GA*	----	31.8	30.1	30.9	1.5	1.5	1.5	
GAH*	----	1.0	<1.0	<1.0	5.3	4.7	5.0	
GAH	Glycine	5.0	<1.0	1.2	<1.1	6.8	6.0	6.4
		10.0	1.4	1.8	1.6	6.8	7.0	6.9
		20.0	3.0	1.4	2.2	7.8	9.2	8.5
		40.0	<1.0	<1.0	<1.0	10.8	10.3	10.5
GAH	L-proline	5.0	1.4	1.8	1.6	6.5	6.0	6.2
		10.0	<1.0	1.2	<1.1	4.5	5.0	4.8
		20.0	<1.0	1.0	<1.0	4.5	5.0	4.8
		40.0	1.0	4.0	2.5	4.4	5.2	4.8
GAH	L-leucine	5.0	1.6	1.4	1.5	4.0	4.5	4.3
		10.0	1.8	1.0	1.4	6.2	5.8	6.0
		20.0	5.8	2.6	4.2	11.8	7.8	9.8
		40.0	5.4	5.8	5.6	12.5	14.5	13.5

GA*	----	22.4	23.4	22.9	0.5	0.8	0.7	
GAH*	----	<1.0	<1.0	<1.0	3.3	4.8	4.1	
GAH*	Glycine	20.0	1.2	1.0	1.1	8.5	8.3	8.4
GAH	L-tyrosine	5.0	<1.0	1.0	<1.0	5.3	6.5	5.9
		10.0	<1.0	<1.0	<1.0	6.5	6.0	6.3
		20.0	1.0	<1.0	<1.0	5.8	5.8	5.8
		40.0	<1.0	1.0	<1.0	6.0	7.3	6.7
GAH	DL-trypto- phane	5.0	<1.0	<1.0	<1.0	5.3	7.3	6.3
		10.0	1.2	1.0	1.1	6.0	5.8	5.9
		20.0	4.0	7.6	5.8	8.5	10.0	9.3
		40.0	3.8	3.0	3.4	8.5	8.0	8.3
GAH	L-lysine	5.0	4.8	2.8	3.8	7.0	5.8	6.4
		10.0	1.2	2.6	1.9	7.0	7.8	7.4
		20.0	1.0	1.0	1.0	7.0	7.0	7.0
		40.0	1.2	1.4	1.3	7.3	8.8	8.1

(Table continued on following page)

TABLE 36 (continued)

Medium	Amino Acid Added (mg/100 ml)		Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)		
			1	2	Ave.	1	2	Ave.
GA*	----		32.2	26.4	29.4	0.5	0.8	0.6
GAH*	----		3.8	1.8	2.8	3.8	5.0	4.4
GAH*	Glycine	20.0	1.2	3.4	2.3	8.3	9.0	8.7
GAH	L-cystine	5.0	7.8	19.0	13.4	9.3	8.5	8.9
		10.0	19.8	23.4	21.6	4.8	8.3	6.6
		20.0	21.4	21.6	21.5	3.0	4.0	3.5
		40.0	22.4	21.2	21.8	3.0	3.0	3.0
GAH	L-serine	5.0	2.8	5.2	4.0	8.8	11.0	9.9
		10.0	1.8	2.2	2.0	11.0	11.0	11.0
		20.0	3.6	3.4	3.5	9.3	13.0	11.2
		40.0	4.4	5.4	4.9	8.5	11.8	10.2
GAH	L-threonine	5.0	3.0	5.2	4.1	7.0	7.3	7.2
		10.0	3.0	4.2	3.6	6.8	6.8	6.8
		20.0	5.8	4.8	5.3	10.0	9.5	9.8
		40.0	5.2	4.4	4.8	11.0	9.3	10.2

GA*	----		37.8	35.4	36.6	1.0	0.8	0.9
GAH*	----		2.6	3.0	2.8	3.8	4.3	4.1
GAH*	Glycine	20.0	2.8	4.2	3.5	5.8	6.5	6.2
GAH	L-alanine	5.0	4.3	2.6	3.0	3.8	2.8	3.3
		10.0	2.8	2.4	2.6	3.8	3.8	3.8
		20.0	2.2	1.8	2.0	3.8	4.0	3.9
		40.0	1.4	2.0	1.7	4.5	4.8	4.6
GAH	DL-phenyl- alanine	5.0	2.0	4.8	3.4	4.0	4.5	4.3
		10.0	1.8	1.4	1.6	4.5	4.5	4.5
		20.0	1.6	2.4	2.0	4.5	4.5	4.5
		40.0	4.0	3.0	3.5	4.3	5.3	4.8
GAH	DL-iso- leucine	5.0	2.6	3.0	2.8	4.0	5.0	4.5
		10.0	3.0	1.8	2.4	4.5	4.3	4.4
		20.0	3.0	2.4	2.7	5.3	5.3	5.3
		40.0	3.2	1.8	2.5	5.3	5.3	5.3

(Table continued on following page)

Medium	Added Amino Acid (mg/100 ml)		Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)		
			1	2	Ave.	1	2	Ave.
GA*	----		30.8	32.8	31.8	1.0	1.0	1.0
GAH*	----		2.6	2.0	2.3	5.0	5.0	5.0
GAH*	Glycine	20.0	1.6	1.8	1.7	10.8	9.7	10.3
GAH	L-methio- nine	5.0	18.0	15.8	16.9	4.8	4.5	4.7
		10.0	22.2	15.0	18.6	4.5	4.8	4.7
		20.0	35.4	30.1	32.8	3.5	5.0	4.3
		40.0	50.4	45.2	47.8	4.5	4.3	4.4
GAH	L-valine	5.0	3.0	3.6	3.3	5.0	5.3	5.2
		10.0	3.0	3.2	3.1	6.0	5.3	5.7
		20.0	2.0	2.0	2.0	5.8	5.5	5.7
		40.0	3.2	3.6	3.4	5.8	6.3	6.1
GAH	Beta- alanine	5.0	1.8	2.6	2.2	6.5	5.8	6.2
		10.0	2.2	2.4	2.3	7.5	8.3	7.9
		20.0	4.2	2.2	3.2	7.5	8.0	7.8
		40.0	2.6	2.6	2.6	7.0	9.0	8.0

* Control media.

than 1.0 mg. to 2.8 mg. per 20 ml. of medium. The riboflavin formed in the GAH control medium was in the range of 4.1 to 5.0 ug. per ml. which was an average of 3.5 ug. per ml. higher than the riboflavin produced in the GA control medium. This would indicate that histidine, at the concentration used in the GAH medium, may have some effect on riboflavin synthesis. If the growth metabolism of A. gossypii is considerably disturbed under these inhibited conditions, the metabolism of glutamic acid and arginine may be forced into other pathways than those for growth, thus some riboflavin may be formed as a result of their changed metabolic products. If such were the case, it would indicate an indirect function of histidine on riboflavin synthesis; however, under these same

conditions histidine may also be assumed to be more directly involved in riboflavin formation.

A total of 15 amino acids were studied, in this series of tests, with respect to their effects on formation of riboflavin in the histidine inhibited cell system. In keeping with the other experiments, growth was also determined in conjunction with riboflavin synthesis. The growth inhibition by histidine was not significantly affected by the amino acids, glycine, L-proline, L-tyrosine, L-alanine, DL-phenylalanine, DL-isoleucine, L-valine or Beta-alanine when added to the GAH media. Assuming the concentration of these amino acids is high enough to overcome any histidine inhibition of their metabolisms, the data would seem to indicate that histidine was not inhibitory to the growth of A. gossypii by blocking the metabolisms of any of these amino acids.

L-leucine, DL-tryptophane, L-serine or L-threonine showed a slight effect in the histidine inhibition when the 20.0 mg. or 40.0 mg. concentrations were added to the GAH medium as shown by the slightly increased growth over the GAH control medium. When only 5.0 mg. of L-lysine HCl was added to the GAH medium, there was a slight increase in growth over the GAH control, but at the higher concentrations of lysine there was no longer any such effect noted.

The sulfur containing amino acids had a large effect on the histidine inhibition of A. gossypii cell growth. The addition of 5.0 mg. or 10.0 mg. of L-cystine to 100 ml. of the GAH medium resulted in 13.4 mg. or 21.6 mg. dry weight of cells, respectively, which was approximately one-half or two-thirds, of the uninhibited cell growth in the GA control medium. L-methionine was even more effective in relieving the histidine

inhibition of growth. The growth in the GAH medium when 20 mg. of L-methionine per 100 ml. was added, equalled that formed in the GA control medium. At the concentration of 40 mg. of methionine per 100 ml. of the GAH medium, the growth in the GAH medium exceeded that of the uninhibited GA control medium by approximately 50 per cent. This strongly suggests that the histidine inhibition may be due to a blocking of the sulfur metabolism of A. gossypii as related to either the formation of methionine or the function of methionine in the growth metabolism.

An examination of the effects on riboflavin synthesis showed that the amino acids L-alanine, DL-phenylalanine, DL-isoleucine, L-valine and L-methionine apparently had no stimulating effect on the synthesis of riboflavin in the GAH medium. It is of interest to note that methionine did not stimulate riboflavin synthesis even though it relieved the histidine inhibition of growth. This would create a condition similar in some respects to growth in the GA medium without histidine; however, the yield of riboflavin is considerably higher than that in the GA control which seems to indicate an effect by histidine on formation of riboflavin. Thus, it would appear that histidine might have a dual effect, one as a growth inhibitor and the other as a stimulator for riboflavin synthesis.

L-proline and L-tyrosine appeared to show a slightly increased riboflavin synthesis over the GAH control when 5.0 mg. of the amino acids was added to 100 ml. of the medium. At higher concentrations of these amino acids, tyrosine showed no further effect but in the case of proline there was a reduction to the value of the control. These amino

acids showed no significant effect on histidine inhibition.

There was a moderate but significant increase in riboflavin formation when L-lysine HCl, Beta-alanine or DL-tryptophane was added to the GAH medium. In the cases of lysine and tryptophane, the synthesis increased as the amount of the amino acid added was increased; however, in the case of Beta-alanine, the maximum amount of riboflavin was formed when 10.0 mg. of the amino acid was added to 100 ml. of the GAH medium and further additions produced no increase.

One of the most striking amino acid effects was shown by L-cystine which stimulated both growth and formation of riboflavin when 5.0 mg. of the amino acid was added to 100 ml. of the GAH medium. As the concentration of cystine was increased the amount of riboflavin formed was shown to decrease, accompanied by an increase in growth of the organism. At the higher concentrations of cystine, the conditions in the medium, with respect to riboflavin synthesis, became similar to those in the GAH control which indicates again the possible dual function of histidine as discussed in the case of methionine.

Large effects on riboflavin formation were demonstrated by the addition of the amino acids glycine, L-leucine, L-serine or L-threonine to the GAH medium. Glycine, leucine or threonine showed a gradual increased synthesis of riboflavin with increasing amounts of the amino acids to the GAH medium. Serine showed increased riboflavin formation at concentrations up to and including 10.0 mg. per 100 ml.; however, at higher concentrations of the amino acid no further stimulation or riboflavin synthesis was shown. Of all the amino acids leucine showed the largest amount of riboflavin formed when it was added at a concentration

of 40.0 mg. per 100 ml. of the GAH medium.

The interrelationships of these amino acids with respect to riboflavin formation and/or growth offer a considerable field for speculation. The effect of cystine and methionine on the histidine inhibition has already been discussed; however, it is worth noting in Figure 1, page 18, that these two amino acids can be related to each other in the amino acid metabolic scheme. The fact that cystine was less effective than methionine in relieving the histidine inhibition may indicate only a partial conversion of cystine to whatever is necessary to completely relieve the histidine inhibition. The initial addition of 5.0 mg. of cystine per 100 ml. may give rise to increased riboflavin because of the possible formation of serine which has been shown to stimulate riboflavin formation; however, the rate of the formation of serine may be a limiting step which would allow only a certain conversion of cystine to serine. Therefore, when increased amounts of cystine are added the large excess present forces the formation of the inhibition relieving substance which in turn inhibits the formation of any riboflavin. This seems to be verified by the fact that increased concentrations of cystine produced an increased inhibition of riboflavin synthesis even though appreciable growth had occurred. On the other hand, methionine probably went directly into the system which relieved the histidine inhibition and as a result riboflavin formation was inhibited as a result of the processes involving the growth of the organism.

The other amino acids, which had a slight relieving effect on the histidine inhibition, have no direct interrelationships with one another or with methionine (see Figure 1). Threonine may have been able to show

its slight relief of inhibition through methionine due to the relationship of the former to homoserine which in turn may form a small amount of methionine.

As a result of the effects of the amino acids on the synthesis of riboflavin, some interesting facts were brought to light. Tyrosine and tryptophane, which are related in Figure 1, did show a stimulating effect on riboflavin formation, with tryptophane being more readily metabolized than the tyrosine. The limited solubility of tyrosine may have been another factor which limited its effectiveness toward riboflavin formation.

The stimulation of riboflavin synthesis by 5.0 mg. of L-proline and the reversal, on the addition of 10.0 mg. of the amino acid was rather difficult to explain. However, in the case of cystine, if proline, at low concentrations, did metabolize to some precursor by a slow reaction, the presence of higher concentration of proline may force the reaction back to glutamate with the resultant formation of inhibitory metabolites of the dicarboxylic acid type reported by Van Lanen, which would effectively reduce the formation of riboflavin.

Lysine and Beta-alanine also showed a moderate stimulation of riboflavin formation which is of interest since both have been shown to be related to aspartic acid which, as has been pointed out previously, may be closely related to riboflavin synthesis. Another possible explanation might be based on the fact that lysine and Beta-alanine have a terminal amino group which might be a factor in riboflavin synthesis. This appears to be the first time that Beta-alanine has been reported in connection with riboflavin synthesis.

Threonine showed an appreciable effect on riboflavin formation and this may result from its relationship to aspartic acid, via homoserine. This was further verified by the slight effect it had on reversing the histidine inhibition since some homoserine may be metabolized to methionine. The fact that isoleucine showed no stimulation of riboflavin synthesis tends to eliminate the formation of isoleucine from threonine as a pathway of effect on riboflavin formation.

In the histidine inhibition medium, the amino acids, serine and glycine, showed a stimulating effect on riboflavin formation which was rather surprising, since in previous experiments serine had not shown any effect on riboflavin synthesis in the GA medium. These data appear to substantiate the fact that serine and glycine are related in their metabolisms. The effect of glycine or serine may indicate a closer relationship to riboflavin formation since the other amino acids which can be formed from glycine and serine do not show any significant stimulation of riboflavin synthesis.

Leucine showed the greatest stimulation of riboflavin formation, with an effect which seems to be separate from any of the other amino acids, since there were no relationships with riboflavin stimulating amino acids noted. The effect of leucine on riboflavin formation may be more closely related to riboflavin synthesis than any effects shown by the other riboflavin stimulating amino acids since it exerted the greatest effect.

An examination of the data seems to show that the amino acids, studied with respect to riboflavin synthesis, can be classified into three groups on the basis of their metabolic interrelationships and effect on

riboflavin formation, namely, those related to aspartic acid, the glycine and serine group and the individual amino acid, leucine. It might be assumed that more than one amino acid may be involved in the formation of the larger amounts of riboflavin.

25. A Study of Special Amino Acid Media. The study of the effect of amino acids on riboflavin formation in the GAH medium offered information which might be used as a guide to develop a synthetic medium containing several amino acids. In previous experiments, the attempts to combine a number of amino acids usually led to a moderate formation of riboflavin or no appreciable yield at all, even though the growth was satisfactory. It was decided to test media containing almost all of the amino acids used in the preceeding experiment using concentrations generally based on those that appeared to be most efficient for riboflavin formation. Since the actual stimulation of riboflavin formation caused by an amino acid is usually based on an increase over a control, the amounts of riboflavin produced above that in the GAH control medium were used in the calculations. From the yields of riboflavin above that in the GAH medium obtained by the addition of each concentration of amino acid to the GAH medium, a yield of riboflavin per mg. of amino acid added was calculated, to determine at which concentration the added amino acid was most efficiently converted to riboflavin. The results of these calculations are shown in Table 37.

An amino acid base medium was prepared from the basal by adding certain concentrations of the amino acids listed in Table 37 as well as concentrations of L-arginine HCl, L-histidine HCl and L-methionine. The concentrations of the amino acids to be added were chosen, in most cases,

TABLE 37

THE RESULTS OF CALCULATIONS TO DETERMINE THE RIBOFLAVIN SYNTHESIZED PER
MILLIGRAM OF AMINO ACID ADDED TO THE GLUTAMIC ACID -
ARGININE - HISTIDINE MEDIUM

Amino Acid	Concentration Added (mg/100 ml)	Riboflavin*	
		(ug/100 ml)	(ug/mg of amino acid)
Glycine	5.0	140	28.0
	10.0	190	19.0
	20.0	350	17.5
	40.0	550	13.7
L-proline	5.0	120	24.0
L-leucine	10.0	100	10.0
	20.0	380	19.0
	40.0	850	21.2
L-tyrosine	5.0	180	36.0
	10.0	220	22.0
	20.0	170	8.5
	40.0	260	6.5
DL-tryptophane	5.0	220	44.0
	10.0	180	18.0
	20.0	520	26.0
	40.0	420	10.5
L-lysine HCl	5.0	230	46.0
	10.0	330	33.0
	20.0	290	14.5
	40.0	400	10.0
L-cystine	5.0	450	90.0
	10.0	220	22.0
L-serine	5.0	550	110.0
	10.0	660	66.0
	20.0	680	34.0
	40.0	580	14.5

(Table continued on following page)

TABLE 37 (continued)

Amino Acid	Concentration Added (mg/100 ml)	Riboflavin*	
		(ug/100 ml)	(ug/mg of amino acid)
L-threonine	5.0	280	56.0
	10.0	240	24.0
	20.0	540	27.0
	40.0	580	14.5
L-alanine	40.0	50	1.3
DL-phenylalanine	5.0	20	4.0
	10.0	40	4.0
	20.0	40	2.0
	40.0	70	1.8
DL-isoleucine	5.0	40	8.0
	10.0	30	3.0
	20.0	120	6.0
	40.0	120	3.0
L-valine	5.0	20	4.0
	10.0	70	7.0
	20.0	70	3.5
	40.0	110	2.8
Beta-alanine	5.0	120	24.0
	10.0	290	29.0
	20.0	280	14.0
	40.0	300	7.5

* Riboflavin in excess of GAH controls in Table 36.

from the data in Table 37 which showed the concentrations giving the highest yield of riboflavin per mg. of amino acid, as recorded in the last column of the table except that none were used above 20 mg. per 100 ml. Certain deviations were made with some amino acids because of prior knowledge of their effect on riboflavin formation and other information related to them. Glycine was added at a concentration of 10 mg. per 100 ml., although the calculations in the above table indicate

that 5.0 mg. per 100 ml. is more efficient, because it was felt that this amino acid was truly involved in riboflavin formation. The concentration changes from the apparently most efficient values of any of the other amino acids were for somewhat similar reasons. The concentration of arginine HCl was increased to 20 mg. per 100 ml. because of the apparent importance of the basic amino acids in the riboflavin synthesizing metabolism. The concentration of histidine HCl and methionine for the base medium was 5.0 mg. per 100 ml. since, in experiments number 19 and 24, respectively, both of these amino acids showed some effect on growth at these concentrations and it was believed that concentrations higher than these might have proved detrimental to riboflavin formation. The composition of the amino acid base medium is shown in Table 38.

TABLE 38

THE AMINO ACIDS ADDED TO THE BASAL IN THE
AMINO ACID BASE MEDIUM

Amino Acid	Concentration (mg/100 ml)	Amino Acid	Concentration (mg/100 ml)
L-arginine HCl	20.0	L-leucine	20.0
L-alanine	10.0	L-lysine HCl	10.0
Beta-alanine	10.0	L-methionine	5.0
L-cystine	5.0	L-proline	5.0
Glycine	10.0	DL-phenylalanine	5.0
L-histidine HCl	5.0	L-serine	5.0
L-threonine	5.0	DL-tryptophane	2.5
L-tyrosine	5.0		

Eight media were prepared by adding different concentrations of the principal nitrogen sources, L-glutamic acid, L-aspartic acid or asparagine to the amino acid base medium without methionine. A peptone control medium was prepared by adding 200 mg. of peptone to 100 ml. of the basal. A solution of methionine, for addition to the media after sterilization, was prepared and sterilized by Seitz filtration. Duplicate flasks of media were sterilized, the required amount of methionine added to all flasks but the peptone controls and all flasks were inoculated with a 28 hour washed cell suspension. The inoculated flasks were incubated on the shaker for 4 days at 28° C. After incubation, the dry weight of cells and colorimetric riboflavin values were obtained. These results and the concentrations of the principal nitrogen sources are shown in Table 39.

At the higher concentrations of the principal nitrogen sources, the riboflavin formed was lower in amount than the peptone medium in all except Medium number 3 which contained asparagine only as the principal nitrogen source. When lower concentrations were used, the yield of riboflavin was approximately the same as that in the peptone medium, except again the asparagine medium, Medium number 6, which showed considerably more riboflavin than the control. The data appear to show that the amino acids closely related to the citric acid cycle caused a decreased synthesis of riboflavin, at the higher concentrations. At the lower concentrations of these amino acids, the riboflavin could be synthesized more readily because there might be a lesser concentration of inhibitory products resulting from excesses of the principal nitrogen sources, since there was little differences in growth. Even though the principal

TABLE 39

THE GROWTH AND RIBOFLAVIN SYNTHESIS ON A SPECIAL AMINO ACID MEDIUM
WITH DIFFERENT CONCENTRATIONS OF PRINCIPAL NITROGEN SOURCES

Medium Number	Principal Nitrogen Source Concentration (mg/100 ml)		Dry Weight Cells (mg/20 ml)			Riboflavin (μ g/ml)		
	Amino Acid		1		Ave.	1		Ave.
			1	2		1	2	
1	Glutamic acid	200	65.2	86.2	75.7	4.3	4.8	4.5
2	Aspartic acid	200	83.8	77.4	80.6	6.5	7.0	6.8
3	Asparagine	200	70.0	71.2	70.6	19.0	21.2	20.1
4	Glutamic acid	50	85.0	79.8	82.4	12.0	20.0	16.0
5	Aspartic acid	50	71.0	68.2	70.0	20.0	10.8	15.4
6	Asparagine	50	74.0	66.6	70.3	59.5	50.0	54.8
7	Glutamic acid Aspartic acid	100 100	70.4	75.8	73.1	6.0	6.5	6.3
8	Glutamic acid Asparagine	100 100	64.4	65.4	64.9	7.0	8.5	7.8
9	Peptone*	200	80.2	85.6	82.9	18.3	11.5	14.9

* Control. Mg. peptone per 100 ml. of basal.

nitrogen source was reduced by one-fourth in three of the media, there was no corresponding growth decrease which had been noted in an earlier experiment when the nitrogen supply was decreased in a medium which lacked amino acids. This was very good evidence that many of the amino acids were being effectively used for the synthesis of cellular material.

The highest yield of riboflavin occurred in Medium number 6 where 0.05 g. of asparagine per 100 ml. of medium was the principal nitrogen source. This yield was more than three times that of the peptone control, even though they both contained approximately the same amount of total nitrogen. In these studies, this was the first instance that riboflavin was produced in a synthetic medium to give a higher yield than the corresponding peptone medium of approximately the same nitrogen content.

26. The Effect of L-serine on Riboflavin Synthesis in Glutamic Acid-Arginine and Glutamic Acid-Arginine-Histidine Media. Previous experiments had shown that serine had no effect on riboflavin formation in the GA medium whereas in the GAH medium it stimulated riboflavin synthesis. Because these results were unexpected, this experiment was performed to verify the previous results. The regular GA and GAH media were prepared with 0.21 g. of glutamic acid per 100 ml. in the GA medium and 40 mg. of histidine HCl per 100 ml. of the GA medium to give the GAH medium. In addition, a second set of media were prepared in which the glutamic acid concentration was reduced to 0.05 g. per 100 ml. which was the concentration in the histidine medium when serine stimulated riboflavin formation. A concentration of 0.01 g. of arginine HCl per 100 ml. was used for all the media. Other media were prepared by adding 28.0 mg. of serine per 100 ml. of the GA and GAH media of both glutamic

acid concentrations. Glycine controls were also prepared by adding 20.0 mg. of glycine per 100 ml. of each GA and GAH media. Duplicate flasks of sterile media were inoculated with a 28 hour washed cell suspension and incubated for 4 days at 28° C. on the shaker. The dry weight of cells and the colorimetric determinations of riboflavin that were made and the concentrations of glutamic acid are shown in Table 40.

TABLE 40

THE EFFECT OF SERINE ON RIBOFLAVIN FORMATION IN GLUTAMIC ACID - ARGININE (GA) AND GLUTAMIC ACID - ARGININE - HISTIDINE (GAH) MEDIA WITH DIFFERENT CONCENTRATIONS OF GLUTAMIC ACID

Medium	Glutamic Acid Added (g/100 ml)	Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)		
		1	2	Ave.	1	2	Ave.
GA	0.21	72.8	62.4	67.6	1.0	1.2	1.1
GAH	0.21	1.6	2.4	2.0	4.0	4.5	4.3
GAGly	0.21	67.0	55.6	61.3	11.0	9.3	10.2
GAHGly	0.21	1.6	2.4	2.0	11.8	10.0	10.9
GASerine	0.21	50.2	46.2	48.2	1.2	1.2	1.2
GAHSerine	0.21	2.6	1.8	2.2	6.8	6.5	6.7
GA	0.05	112.9	12.2	12.6	0.5	0.5	0.5
GAH	0.05	1.4	1.0	1.2	4.3	4.0	4.2
GAGly	0.05	12.1	9.9	11.0	5.0	8.5	6.8
GAHGly	0.05	1.0	1.0	1.0	7.5	8.3	7.9
GASerine	0.05	11.4	5.0	8.2	0.5	0.8	0.7
GAHSerine	0.05	1.0	1.0	1.0	5.3	5.3	5.3

The results of this experiment confirm the previous findings and did show that serine stimulated riboflavin formation in the GAH medium, whereas, it did not show this effect under growth conditions in the GA medium. The data showed that glycine was more effective than serine in stimulating riboflavin formation in either media which may indicate that in the GAH medium with serine, the serine was converted to glycine rather than the reverse. In the rapidly growing medium the concentration of glycine that may be formed from serine may never reach the value necessary for riboflavin formation but in the inhibition medium, a sufficient concentration of glycine may be reached since the glycine can not be used for growth and thus is available for riboflavin synthesis. These results further establish the usefulness of the histidine inhibited medium for studies of riboflavin synthesis, and that the addition of serine to media may also be helpful.

27. The Effect of Different Concentrations of Asparagine on Growth and Riboflavin Formation in a Peptone Medium. Asparagine, the amide of aspartic acid, may be associated with the biosynthesis of riboflavin since previous experiments have shown that the greatest stimulation of riboflavin formation occurred in media containing this compound. In order to test this possibility further, different concentrations of asparagine were added to a peptone medium composed of 0.2 g. of peptone per 100 ml. of the basal. Duplicate flasks of the sterilized media were inoculated with a 28 hour washed cell suspension and incubated on the shaker for 4 days at 28° C. The dry weight of cells and the colorimetric riboflavin values were determined. These values and the concentrations of asparagine added are shown in Table 41.

TABLE 41

THE EFFECT OF DIFFERENT CONCENTRATIONS OF ASPARAGINE ON GROWTH
AND RIBOFLAVIN FORMATION IN A PEPTONE MEDIUM

Asparagine Added (mg/100 ml)	Dry Weight Cells (mg/20 ml)			Riboflavin (ug/ml)		
	1	2	Ave	1	2	Ave.
0*	88.2	78.4	83.3	18.5	13.0	15.8
2.5	82.8	79.6	81.2	36.3	33.5	34.9
5.0	83.0	84.8	83.9	34.6	30.0	32.3
10.0	65.0	80.2	72.6	27.6	22.0	24.8
20.0	94.2	66.6	80.0	17.0	25.6	21.3
40.0	86.2	78.2	82.2	11.0	8.8	9.9

* Peptone control.

The addition of only 2.5 mg. of asparagine per 100 ml. of the peptone medium resulted in riboflavin yield which was more than double that found in the peptone medium alone. These data further substantiate the relationship of asparagine to the formation of riboflavin in A. gossypii. Asparagine may itself be related to the synthesis or it may be involved in the formation of some other compound which is involved in the synthesis process. The formation of riboflavin was inhibited when 40 mg. of asparagine was added to 100 ml. of the peptone medium, which was probably the same sort of an effect which was noted when the glutamic acid medium was added to peptone. The addition of even high concentrations of asparagine to the peptone medium failed to cause any significant change in the growth of the organism which was further

evidence that asparagine must function very closely to the riboflavin synthesizing metabolism of A. gossypii. In studying biosynthesis pathways for riboflavin in media supporting growth and riboflavin formation, further research on addition of asparagine might yield useful information.

IV. SUMMARY AND CONCLUSIONS

Studies on the growth and riboflavin metabolism of Ashbya gossypii were undertaken in an effort to gain more facts pertaining to the mechanism of riboflavin synthesis in microorganisms and thereby bring about a more complete understanding of the pathway and intermediates participating in the synthesis.

During these studies the circular chromatographic technique was introduced and its utility demonstrated in conjunction with the rapid, qualitative chromatographic separation of unknown mixtures. The observed R_f values of amino acids and peptides were reported for the n-butanol-acetic acid-water and pyridine-tertiary amyl alcohol-water solvents employing the circular chromatographic technique. The use of this technique in relation to further studies on the metabolism of A. gossypii is indicated.

A colorimetric method for the analysis of riboflavin was developed in order to provide a more rapid assay of the results obtained in the different experiments. This method, although not as accurate as the fluorometric method, did give indications that it could be refined further, for use as a quantitative method for riboflavin determinations, especially in culture media for the study of riboflavin synthesis, where there is only a small amount of interfering substances present. A check of the method showed reasonable recoveries of added riboflavin in unknown samples could be obtained, further demonstrating that the method could be used

for approximate estimations of riboflavin formation.

The riboflavin formation by A. gossypii in a yeast extract-peptone medium was shown to occur almost entirely after the organism had reached maximum growth. The formation of riboflavin in the medium was also accompanied by an increase in pH of the medium, the pH having reached a minimum value at approximately the age of maximum growth. During the initial phases of growth, the organism must apparently have to produce some special conditions and/or intermediates for the formation of riboflavin, since the morphology of the organism showed marked changes during this period.

The complex nitrogen source, peptone, was fractionated on a powdered cellulose column, using a n-butanol-acetic acid-water solvent, and the resulting fractions tested for growth and riboflavin formation with A. gossypii. All, except one, of these fractions gave good growth of the organism; however, only one fraction gave good growth and allowed the formation of appreciable riboflavin. The chromatographic analysis of the hydrolyzate of this riboflavin stimulating fraction indicated that there was a predominance of basic amino acids present, as well as dicarboxylic amino acids. A study of the possible amino acids present in this fraction resulted in the development of a synthetic medium, containing glutamic acid and arginine as the nitrogen sources, which supported good growth of A. gossypii but allowed no significant riboflavin formation.

Since the inocula for these studies were prepared from cells grown on yeast extract-peptone stock agar slants, the possibility of substances being carried over in the inoculations was investigated by a series of

studies involving the serial transfer of cells, on solid and in liquid, natural and synthetic media. It was apparent that some factor or factors were being carried over from the yeast extract-peptone agar slant in the first transfer; however, in subsequent transfers the factor or factors seemed to have been diluted out to a point where there was little effect. Although this carry over effect cannot be overlooked, the effect of it should be rather constant under any one particular set of experimental conditions and the results of a particular experiment can be discussed with respect to added known factors.

Aqueous cell extracts of A. gossypii, obtained by grinding the cells in a small amount of distilled water with ground glass and sand, were shown to stimulate riboflavin synthesis in a synthetic medium containing glycine. This stimulation effect was shown to be present principally in older cells, since the effect was demonstrated with 132 hour cells but could not be demonstrated with 8 $\frac{1}{2}$ hour cells from the same growth medium. The possibility of enzymes or enzyme systems being introduced in the aqueous cell extract was eliminated by autoclaving of the media to which the extracts had been added. The chromatographic separation on filter paper strips and the subsequent testing of the fractions found indicated that the factor or factors in the aqueous cell extract, which stimulated riboflavin formation, did not migrate in the butanol-acetic acid-water solvent system.

The addition of relatively large concentrations of glutamic acid or asparagine to a peptone medium resulted in the decreased formation of riboflavin and a corresponding increased growth. The results of many of the experiments show this inverse relationship between growth and

riboflavin formation which probably indicates that conditions for good growth of the organism might not be conditions for good riboflavin synthesis.

Other nitrogen sources were tested for growth and riboflavin formation with A. gossypii and it was found that NH_4NO_3 , KNO_3 , $(\text{NH}_4)_2\text{SO}_4$, ammonium acetate, ammonium citrate and urea would not serve as single nitrogen sources for either growth or riboflavin formation. The presence of relatively high concentrations of nitrate ions in a peptone medium was found to be inhibitory to the formation of riboflavin by A. gossypii, but apparently had no effect on the growth of the organism.

In synthetic media with glutamic acid, aspartic acid or asparagine as the principal nitrogen sources, the addition of glycine resulted in an increased formation of riboflavin, especially in the presence of aspartic acid or asparagine. The addition of L-serine or DL-threonine, instead of glycine, to the same media resulted in no increased riboflavin formation. Under the conditions used, it appeared that the synthesis of riboflavin was more closely associated with aspartic acid than glutamic acid and that glycine was associated in some way with the formation of riboflavin.

The addition of L-histidine HCl to a synthetic medium containing glycine had a varied effect on growth and riboflavin formation by A. gossypii. In low concentrations, 2.5 mg. to 10.0 mg. per 100 ml., it had a stimulatory effect on growth which was accompanied by a slight decrease in formation of riboflavin; however, at higher concentrations, 25.0 mg. to 40.0 mg. per 100 ml., it inhibited the growth of the organism but did not inhibit the formation of riboflavin. By this inhibition effect,

the growth metabolism of the organism and the riboflavin synthesizing metabolism had been apparently separated. When imidazole, a compound making up part of the histidine molecule, was added, in the same approximate concentrations as histidine, to the synthetic media without histidine, no inhibition of the organism was noted.

A system of histidine inhibited cells of A. gossypii was used to study the effects of 15 amino acids on riboflavin synthesis and growth. The amino acids, glycine, proline, tyrosine, alpha-alanine, beta-alanine, phenylalanine, isoleucine or valine had no effect on the growth of the organism in the presence of histidine; however, the amino acids leucine, tryptophane, lysine, serine and threonine did relieve the histidine inhibition slightly, usually at the highest concentrations used. The amino acids cystine and methionine relieved the inhibition of histidine which seemed to relate the inhibition by histidine to the metabolism of the sulfur containing amino acids. The amino acids alpha-alanine, phenylalanine, isoleucine, valine and methionine showed no effect on riboflavin synthesis in the histidine inhibited cell system, whereas proline and tyrosine showed a slight effect and cystine, lysine, tryptophane and beta-alanine showed a moderate effect. The greatest effect on riboflavin formation was shown by the amino acids glycine, serine, threonine and leucine, with leucine showing the most effect of the four amino acids listed.

A synthetic medium containing 17 different amino acids was found to allow the production of considerable riboflavin and permitted good growth of A. gossypii. The riboflavin formed was over three times that formed in a peptone medium of approximately the same percentage nitrogen.

This study of the metabolism of riboflavin in Ashbya gossypii has pointed out that the biosynthesis of riboflavin is related to many factors. It appears that the synthesis of riboflavin can be placed into three different group functions as follows: one, aspartic acid and those compounds related to it, two, glycine and serine, and three, leucine.

Various conditions have been developed which permit the study of the metabolism of A. gossypii under different circumstances, namely, growth with little or no riboflavin formation, growth with different degrees of riboflavin formation and no growth with riboflavin formation. It is evident that further work, such as, studies of radio active tracers, short time chromatographic analyses, amino acid antagonists, the mechanisms of histidine inhibition, etc., must be done in order to more completely understand the pathways of riboflavin synthesis in microorganisms.

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BIOGRAPHICAL ITEMS

The writer was born on July 12, 1921 in Cincinnati, Ohio. His early education was obtained through the Public School System of Cincinnati, Ohio. He served as a commissioned officer with the United States Marine Corps during World War II. He pursued his undergraduate studies at the University of Cincinnati, The University of Tampa and the University of Florida, receiving the Bachelor of Science Degree, with honors, from the University of Florida in August, 1949.

He continued graduate studies in Biochemistry at the University of Florida and received the Degree of Master of Science in February, 1951. He attended Purdue University from February, 1951 to August, 1952, doing graduate work in bacteriology. He returned to the University of Florida in September, 1952, as an Assistant in Research at the Engineering and Industrial Experiment Station. He became a teaching assistant in the Chemistry Department of the University of Florida in February, 1954, serving in that capacity until he was awarded the Tennessee Eastman Kodak Research Fellowship in September, 1955. After his return to the University of Florida in 1952, he pursued graduate studies in biochemistry leading to the Degree of Doctor of Philosophy in Chemistry.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of the committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June 4, 1956

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