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Biosynthesis of Glutamic Acid

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The *Butler University Botanical Studies* journal was published by the Botany Department of Butler University, Indianapolis, Indiana, from 1929 to 1964. The scientific journal featured original papers primarily on plant ecology, taxonomy, and microbiology. The papers contain valuable historical studies, especially floristic surveys that document Indiana's vegetation in past decades. Authors were Butler faculty, current and former master's degree students and undergraduates, and other Indiana botanists. The journal was started by Stanley Cain, noted conservation biologist, and edited through most of its years of production by Ray C. Friesner, Butler's first botanist and founder of the department in 1919. The journal was distributed to learned societies and libraries through exchange.

During the years of the journal's publication, the Butler University Botany Department had an active program of research and student training. 201 bachelor's degrees and 75 master's degrees in Botany were conferred during this period. Thirty-five of these graduates went on to earn doctorates at other institutions.

The Botany Department attracted many notable faculty members and students. Distinguished faculty, in addition to Cain and Friesner , included John E. Potzger, a forest ecologist and palynologist, Willard Nelson Clute, co-founder of the American Fern Society, Marion T. Hall, former director of the Morton Arboretum, C. Mervin Palmer, Rex Webster, and John Pelton. Some of the former undergraduate and master's students who made active contributions to the fields of botany and ecology include Dwight. W. Billings, Fay Kenoyer Daily, William A. Daily, Rexford Daudenmire, Francis Hueber, Frank McCormick, Scott McCoy, Robert Petty, Potzger, Helene Starcs, and Theodore Sperry. Cain, Daubenmire, Potzger, and Billings served as Presidents of the Ecological Society of America.

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BIOSYNTHESIS OF GLUTAMIC ACID

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ABSTRACT

Tan, W. C. (Dept. of Biochemistry, Indiana U., School of Medicine, Indianapolis, Indiana, U.S.A.), and **Bernard Malin.** Biosynthesis of Glutamic Acid. Butler U. Bot. Studies 14(2):89-103. 1964.—An unknown microorganism was isolated from the soil by using defined selective medium. It could synthesize glutamic acid from glucose and urea. When subjected to repeated ultra-violet light and X-ray irradiations, and after process of screening, the organism had increased the yield of glutamic acid biosynthesis up to 10 mgm per ml of broth. The pathway of glutamic acid biosynthesis is unknown. Evidences indicated that the TCA cycle was probably not involved. The microorganism could be a new species of *Micrococcus*.

Glutamic acid may be produced either by chemical or fermentation method. By the chemical method, hydrolyzed protein such as soybean cake or wheat gluten is used. However, the product obtained is always in the DL-form or racemic form. Separation of the D and L forms is not only difficult but also time consuming and expensive. On the other hand, biosynthesis of glutamic acid yields exclusively the L form. A few microorganisms are known to possess this biosynthetic ability (1). All of these microorganisms have been isolated by the fermentation industry companies in Japan. The method of isolation has not been revealed. It is known that only after long years of research a strain which can synthesize glutamic acid was obtained.

Glutamic acid is an important flavoring agent which has the largest commercial demand among the various amino acids (2). Therefore special effort has been devoted to the production of L-glutamic acid especially by fermentation method which is now on a commercial scale.

Many microorganisms can synthesize various amino acids in small amounts in culture media. But to obtain a particular organism which can produce

mainly glutamic acid in comparatively good yield, is quite a task. The organism must be able to use carbohydrates and nitrogenous compounds as starting materials for the biosynthesis of glutamic acid. We hereby report the method of screening, isolation, and improving the strain for higher yield of glutamic acid.

The main intent of this study was to isolate an organism which can synthesize glutamic acid from various carbohydrates and compounds with amino group. To do that, screening of microorganisms from various origins and in different media has been performed. We also speculated on the biosynthetic pathway by which glutamic acid is synthesized.

EXPERIMENTS AND RESULTS

Isolation and Testing. Forty 50 ml. flasks each containing 10 ml. of broth were inoculated with different inocula. For the sources of inocula and types of media see TABLE 1. The conditions for the culturing were: room temperature, aerobic, stationary. Six sources of inocula and eight types of media constituted 48 different combinations as indicated by TABLE 2. The composition of media is given in TABLE 3.

After 6 days of stationary culture transfer of the organisms was made to fresh liquid broths and incubated for another 6 days. This was done in order to allow the desired organisms to adapt and to multiply in the individual environment. Observations were made frequently and all the broths were

TABLE 1. INOCULA AND MEDIA USED IN THE EXPERIMENTS

INOCULA	
No.	Name
A	Fermented boiled soybean
B	Faeces of Guinea pig
C	Fresh brown soil
D	Fresh yellow soil
E	Fermented soybean juice
F	Decayed meat
MEDIA	
No.	
1	10% Casein hydrolysate broth
2	10% Sodium citrate broth
3	10% Glucose broth
4	1% alpha-ketoglutarate broth
5	10% Soybean meal broth
6	10% Sucrose broth
7	10% Distillers solubles broth
8	10% Molasses broth

TABLE 2. FINAL pH DETERMINATION

	MEDIA (1) Casein	(2) Citr.	(3) Glucose	(4) Ketoglu.	(5) Soybean	(6) Sucro.	(7) Distil.	(8) Molas.
A. Soybean	8	7	5	9	8	5	7	5
B. Faeces guinea pig	7	9	5	9	9	5	7	4
C. Brown soil	7	8	5	9	7	5	7	5
D. Yellow soil	7	9	5	9	5	5	7	4
E. Soybean juice	8	8	5	9	9	5	7	6
F. Decayed meat	8	7	5	9	8	5	7	4

TABLE 3. HETEROTROPHIC AND AUTOTROPHIC MEDIA

BASAL MEDIUM

Carbon Sources*	10.00%
Urea	1.00%
Meat Extract	0.20%
Peptone	0.20%
K_2HPO_4	0.10%
$MgSO_4 \cdot 7H_2O$	0.02%

pH — 7±
Sterilized

AUTOTROPHIC MEDIUM

$MgSO_4 \cdot 7H_2O$	0.20 g.
K_2HPO_4	1.00 g.
$FeSO_4 \cdot 7H_2O$	0.05 g.
$CaCl_2$	0.02 g.
$MnCl_2 \cdot 4H_2O$	0.002 g.
$NaMoO_4 \cdot 2H_2O$	0.001 g.
NH_4Cl	1.50 g.
$CaCO_3$	5.00 g.
H_2O q.s.	1,000.00 ml.

pH—7.5

*Carbon Sources:

(1) Casein hydrolysate
(2) Sodium Citrate
(3) Glucose
(4) alpha-ketoglutarate 1%
(5) Soybean meal
(6) Sucrose
(7) Distillers Solubles
(8) Molasses

found to sustain growth of the organisms. After a series of transfers only the predominant strains would multiply. It was found that only a few strains of organisms could grow in media with citrate or alpha-ketoglutarate as the chief carbon source. All other media designed easily supported growth. The media used were designed for selective purposes.

Inoculum A was obtained by boiling 10 gms. of soybean with water until softened. On cooling it was then inoculated with a suspension of fresh, uncultivated soil. After 2 weeks incubation the mixed cultures were used for further inoculations.

As seen from TABLE 2, organisms grown in the carbohydrate media rendered the pH on the acid side while those grown in non-carbohydrate media yielded either neutral or basic pH values.

After many transfers the organisms in the various media were tested for any glutamic acid synthesis ability. Here paper chromatography technique was employed throughout. The developing solution was made of a mixture of n-butanol, glacial acetic acid, and water to the ratio of 4:1:1 by volume. Ninhydrin solution of 0.2% was used for color reaction in order to detect the presence of glutamic acid. On the same paper chromatogram, pure glutamic acid was used as the control standard for comparison and estimation of the concentration with the unknowns. The results for glutamate synthesis tests are given in TABLE 4. One single plus sign indicates that only a small trace of glutamic acid was found on the paper chromatogram.

TABLE 4. RESULTS OF GLUTAMIC ACID BIOSYNTHESIS DETERMINATION BY PAPER CHROMATOGRAPHY

	MEDIA							
	(1) Casein	(2) Citr.	(3) Gluc.	(4) KGT.	(5) Soybean	(6) Sucro.	(7) Distil.	(8) Molas.
Control	+++	−	−	−	−	−	−	−
A. Soybean	+++	+	−	−	+++	+	−	+
B. Faeces Guinea Pig	+++	−	−	+	++	+	−	−
C. Brown Soil	+++	−	−	+	++	−	+	+
D. Yellow Soil	+++	−	−	−	++	−	−	−
E. Soybean Juice	+++	−	+	−	++	+	−	−
F. Decayed Meat	+++	+	−	−	+	−	−	−

It is apparent that casein broth yielded a high quantity of glutamic acid and other amino acids. This is probably due to the degradation of protein molecules during the process of autoclaving. Soybean meal broths also yielded glutamic acid and other amino acids, but here it was found without question that this was due to degradation of the protein molecules by the microorganisms. The use of these two media was intended to illustrate that glutamic acid could be obtained by microbial degradation rather than by biosynthesis from the carbohydrate and an amino group. Other organisms that can degrade soybean to yield glutamate are numerous. A few have been tested in this study: These are *Bacillus subtilis, B. megaterium, Serratia marcescens* and a number of fungi.

This experiment was carried on therefore using those organisms that can synthesize glutamate from carbohydrate and an amino group donor compound, i.e., urea.

At this stage the organisms in each broth could still be a mixed culture. Streak plate technique was used to further purify the individual organisms isolated for subsequent testings.

From the plates hundreds of colonies were isolated and tested individually for any glutamate synthesis ability. After a long series of screening only eight strains from different colonies were found to be able to produce only a faint trace of glutamic acid as shown on the paper chromatogram. These organisms are obtained from the following combinations: A5, A6, A8, B6, C4, C5, C7, C8, of TABLE 4.

Radiation Method. X-ray and ultraviolet light were used alternately to irradiate the isolated organisms. General spectrum X-ray was used. A total dose of 270 roentgen was administered during each experiment. The target was placed at zero distance from the source of irradiation. This total dose given was divided into three doses of 90 r each operating at 300 m.a. second, and 90 K.V.P. Cultures grown in 10% glucose broth less than 24 hours were exposed to the source of X-ray, and later transfer was made to the streak plates prepared. The various survivors were allowed to grow into colonies and were subsequently assayed for any sign of glutamate synthesis. The strains used in the experiment were isolated from A(5,6,8), B(6), and C(4,5,7,8) as referred to in the preceding TABLES. In general, the survival rate was found to be inversely proportional to the number of doses.

Paper chromatography work indicated that 2 strains labeled as A3 and B3 after the X-ray irradiation did definitely increase yields of glutamate synthesis. The reaction spots on the paper chromatogram after addition of ninhydrin were no longer faint traces but appeared as definite blue areas of glutamic acid. In addition to this a slight trace of an unknown amino acid was also found on the same chromatogram.

Following the X-ray experiments, the two strains isolated from above

TABLE 5. OBSERVATION AFTER 6 DAYS OF FERMENTATION[3]

Organisms	Media	Glutamate	Gas	Final pH	Growth	Others
A3-IV	1% alpha-ketoglut.	—	—	8	+	Sediment
A3-IV	10% citrate.	—	—	7	+	
A3-IV	10% glucose + yeast extract (0.2%)	+	+	5	+	Sediment
A3-IV	10% glucose — yeast extract (0.2%)	—	+	7	+	Sediment
A3-III[1]	1% alpha-ketoglut.	—	—	8	+	Sediment
A3-III[1]	10% citrate	—	—	7	+	
A3-III[1]	10% glucose + yeast extract (0.2%)	+	+	7	+	Sediment
A3-III[1]	10% glucose — yeast extract (0.2%)	—	+	7	+	Sediment
Jamaica soil	1% alpha-ketoglut.	—	—	8	+	Sediment
Jamaica soil	10% citrate	—	—	7	+	
Jamaica soil	10% glucose + yeast extract (0.2%)	—	+	5	+	Sediment
Jamaica soil	10% glucose — yeast extract	—	+	5	+	Sediment
A3-IV 7-24	1% alpha-ketoglut.	—	—	7	—	Sediment
A3-IV 7-24	10% citrate	—	—	7	—	Sediment
A3-IV 7-24	10% glucose + yeast extract (0.2%)	—	—	5	+	Sediment
A3-IV 7-24	10% glucose — yeast extract		—	7	—	Sediment
A3-III[2]	1% alpha-ketoglut.	—	—	8	+	Sediment
A3-III[2]	10% citrate extract (0.2%)	—	—	7	+	
A3-III[2]	10% glucose — yeast extract	—	+	7	+	Sediment

1. A3-III pretreated with 40 mg.10 ml. Sulfa drug of m-amino phenol derivative.
2. A3-III exposed to 1 mc of Co-60 for one week.
3. All the controls (4 media without inocula) had pH-7 and were negative for glutamate, gas, growth and sediment tests.

were now subjected to ultraviolet light irradiation. Here the effects were non-ionizing and so plated organisms, instead of broth cultures, were employed. The wavelength of the UV light was in the neighborhood of 2,600 A. The nucleic acids strongly absorb ultraviolet light at this wavelength due to the presence of pyrimidine (15). The room was kept dark while the organisms were exposed to the irradiation and the organisms were not allowed to come into contact with visible light for 24 hours after the experiment in order to prevent photoreactivation (16). In this UV method, young cultures less than 24 hours old in 10% glucose broth with cytidine 10 Mcg./1 were exposed to the radiation for a period of 5 minutes.

After the irradiation 2% casamine acid was added to the medium. This

was done with the hope that the survivals might incorporate the nutrients and therefore synthesize new or different deoxyribonucleic acid in the nucleus of the cells and thus become mutant strains. The distance between the light source and the target was about 9 inches. After a series of transfers, subsequent assay showed that only the strain which is now designated A3-1, which was isolated from the survivors, still retained the ability to synthesize glutamic acid. There was no indication of increase in yield.

Using the A3-1 strain, radiation experiments of X-ray and UV light were performed for the second time. This time 270 r was again given and the nsual assay was performed to determine the glutamate synthesis ability. This was followed by ultraviolet light irradiation in which the length of exposure period was 15 minutes. Again, from the many survivals tested, a new strain was chosen. This new strain designated as A3-11 gave a final pH of 5 and produced gas after one week's fermentation. An unknown amino acid other than glutamic acid was still found to be present. However, this was present in such an extremely small amount that the paper chromatogram showed only a faint trace. This new strain showed a definite indication of glutamic acid synthesis. Colonies that were found to be unable to synthesize glutamate were discarded.

Alternately, the A3-11 was subjected to further irradiation experiments. Since the X-ray dosage is accumulative, the strain at this time had received a total of 810 r and a total of 45 minutes of exposure time to UV light radiation. The last dose given consisted of the usual 270 r X-ray and 25 minutes of UV light radiation. The same procedure was repeated for testing and an organism isolated now called A3-111 possessed the same ability for glutamate synthesis.

At this stage the surviving organisms after radiation showed very slow rates of growth, but the addition of 0.2% yeast extract to the broth restored the growth rate to normal. Exposure of organisms to 1 mc of radioactive cobalt-60 also retarded the growth rate but glutamate synthesis ability was not noticeably altered. The exposure time ranged from 24 hours to one week. The shielding was built of lead bricks and the plates of growing organisms were placed at almost zero distance to the radioactive source. The Co-60 radiation method was found to yield inferior results as compared with X-ray and UV light; X-ray and UV light yielded more satisfactory results.

Strain AS-111 was now subjected to a final 30 minutes of UV light radiation. After the irradiation, only several surviving colonies were found among which only 1 colony was discovered to possess glutamate synthesis capability in apparent high yield. This latest strain was called A3-IV (or D2a-7-28); see TABLE 4. Faint traces of other unknown amino acids could still be detected but the amounts were so negligible that estimation was rendered difficult.

TCA-Cycle Study. From TABLE 6, one can see that media using TCA-cycle intermediates alpha-ketoglutarate and citrate as the chief carbon sources were used to determine the cycle's mechanism with regard to the organisms isolated. It is seen that no glutamate was produced. Either by anaerobic or aerobic method the results obtained proved to be negative. Therefore, from these experiments it can be concluded that TCA-cycle intermediates are not the immediate precursors as far as glutamate synthesis is concerned (17).

TABLE 6. RESULTS FROM TCA CYCLE STUDY

Organisms	Media*	Glutamate	Final pH	Gas	Growth	Oxidation
A3-IV	3a	±	5	+	+	anaerobic
A3-IV	3b	—	5	+	+	anaerobic
A3-IV	2	—	7	—	+	anaerobic
A3-IV	4	—	8	—	+	anaerobic
Saccharomyces ellipsoideus	3a	±	6	±	+	anaerobic
ellipsoideus	3b	—	7	—	—	anaerobic
ellipsoideus	2	—	7	—	—	anaerobic
ellipsoideus	4	—	7	—	—	anaerobic
A3-IV	3a	+	7	+	+	aerobic
A3-IV	3b	—	6	+	+	aerobic
A3-IV	2	—	7	—	+	aerobic
A3-IV	4	—	8	—	+	aerobic
Saccharomyces ellipsoideus	3a	—	6	+	+	aerobic
ellipsoideus	3b	—	6	—	—	aerobic
ellipsoideus	2	—	7	—	—	aerobic
ellipsoideus	4	—	8	+	+	aerobic
none	3a	—	7	—	—	aerobic
none	3b	—	7	—	—	aerobic
none	2	—	7	—	—	aerobic
none	4	—	7	—	—	aerobic

* 3a—10% glucose broth + 0.2% yeast extract
3b—10% glucose broth — 0.2% yeast extract
2 —10% citrate broth
4 — 1% alpha-ketoglutarate broth

Cell Free Extract Study. In this study as illustrated in TABLE 7, A3-IV strain was used to provide the supernatant (18) (19). The TCA-cycle intermediates were again used for anaerobic and aerobic fermentation. Here aerobically the results were negative for glutamate synthesis. It is worth mentioning on the other hand that in the anaerobic system citrate seemed to

TABLE 7. CELL FREE EXTRACT STUDY ON TCA CYCLE MECHANISM

Organism used	Media*	Glutamate	Final pH	Gas	Oxidation
A3-IV	3a	+	7	—	aerobic
A3-IV	3b	+	7	—	aerobic
A3-IV	2	—	8	—	aerobic
A3-IV	4	—	8	—	aerobic
A3-IV	3a	+	5	+	anaerobic
A3-IV	3b	±	6	+	anaerobic
A3-IV	2	±	7	—	anaerobic
A3-IV	4	—	8	—	anaerobic
A3-IV	none				
A3-IV	none	—	7.8	—	anaerobic
A3-IV	none	—	7		aerobic

* 3a = 10% glucose broth + 0.2% yeast extract
3b = 10% glucose broth
2 = 10% citrate broth
4 = 1% alpha-ketoglutarate

show a barely perceptible amount of glutamate catalyzed by supernatant enzymes of the cells.

Estimation of Glutamate Yield by Means of Paper Chromatography. To estimate the yield, standard solutions glutamic acid ranging from a concentration of 10 mg./ml. to a concentration of 1 mg./ml. were used for comparison with the microbial yields (TABLE 8). The criteria for comparison are color intensity and radius of the spot. In this test, all conditions remained the same except that the cultures were incubated for 3 days while in a reciprocal shaker at 100 r.p.m. Shaking was found to be more advantageous than stationary culture due to an increase in the yield; this resulted in an increase in oxygen consumption. TABLE 8 shows the results of yields by various strains. Judging from the yield differences it can safely be concluded that prolonged radiation can increase the yield over original isolates capable of gluamate synthesis although this may take an unusually high dosage to achieve the goal desired.

Optimum Conditions of Fermentation. The various organisms isolated were subjected to a series of tests to determine the optimum factors for growth and glutamate synthesis. Four different media, glucose, distiller's soluble, molasses, and sucrose broths were used. Glucose broth was the only one found that the test organisms isolated could use for biosynthesis of glutamate. A3-1 to A3-IV showed the same results.

Glutamate was detected within 12 hours after fermentation began. The yield was at its maximum during four to six days after the fermentation

TABLE 8. THE GLUTAMIC ACID YIELD ESTIMATION[2]

Organisms	Medium[1]	Glutamate Yield	Final pH	Growth
Sacch. ellip.	3a	—	5	+
Aceto. subox.	3a	—	6	+
A3-I	3a	1 mg./ml.	7	+
A3-II	3a	0.50-0.70 mg./ml.	7	+
A3-III	3a	—	5	+
A3-IV	3a	0.75 mg./ml.	7	+

1. 3a = 10% glucose + 0.2% yeast extract.

2. Incubation was done at room temperature with 10 ml. broth in 50 ml. flask under aerobic and reciprocal shaking of 100 rev. per minute. For testing, the broth was filtered and paper chromatography technique was used. Radius and intensity of each unknown were compared with a series of knowns.

period. After this period no increase in the yield was noted. The optimum time for maximum yield would therefore seem to be about 5 days.

Growth and glutamate synthesis were observed in glucose broths having a pH value around 7. No growth was seen at pH values other than 7. Organisms failed to grow at pH values of 1, 3, 5, 9, 11, 13.

Four different temperatures were used for fermentation. All showed signs of growth and glutamate synthesis. But the growth and yield of glutamate were best at 35°C and least at 18°C. At 25°C the differences were not great.

To determine the optimum concentration of glucose, two fermentation periods of 3 and 5 days at temperatures of 30°C and 37°C were employed. Reciprocal as well as rotary shakers were employed. All these factors did not influence to any great extent the yield of glutamate synthesis. The optimum concentrations were 8 and 10% of glucose as the chief carbon source. The yield was low when 1, 3, and 5% of glucose were used. In the experiment using 8% and 10% glucose over a 5-day fermentation period at 37°C, by rotary shaker, yields as high as 1-1.5 mg. per ml. were obtained. The final pH was 4-5.

It should be pointed out that all the organisms isolated in A3 series experiments produced cloudy sediments and gas in stationary culture. In reciprocal or rotary shaking fermentation studies the final broths were without cloudy sediments.

Identification of the strains. All the strains isolated had undergone the determinative procedures for identification. All of them were first thought to be *Micrococcus caseolyticus*. But later in the biosynthesis study *M. caseolyticus* obtained from American Type Culture Collection was found to be unable to synthesize glutamate under the same fermentation conditions (TABLE 9). Further, gas formation and cloudy sediments were absent dur-

TABLE 9. MICROCOCCUS CASEOLYTICUS FERMENTATION STUDIES[2].

Media[1].	Glutamate Synthesis	Final pH	Gas	Growth	Sediment
3a	—	5	—	+	—
3b	—	3	—	+	—
3c	—	5	—	+	—
4	—	8	—	+	—
2	±	7	—	+	—
Autotrophic	—	7	—	—	—

1. 3a = 10% glucose broth + 0.2% yeast extract
   3b = 10% glucose broth — yeast extract
   3c = 10% glucose broth as listed in Table 3
   4 = 1% alpha-ketoglutarate broth
   2 = 10% citrate broth
2. Fermentation time was 6 days

ing stationary culture. This established the fact that the isolated A3 organisms are at least not typical of *M. caseolyticus*.

Results from determinative procedures are listed below. Cells 2 days old at 30°C were used.

Cell Morphology: Oval to circular (cocci), 0.3-0.6 microns, no flagella, no motility, no spores.

Staining Characteristics: Gram variable, non-acid fast, capsule stain negative, and granule stain positive.

Nutrient Agar Colonies: Form: circular; elevation: convex; surface: shiny and smooth; margin: even; density: slimy; internal structure: moist; chromogenesis: ivory.

Nutrient Agar Stroke: Amount of growth: abundant; form: slight spreading and raised edge; consistency: slimy; density: moist; chromogenesis: ivory.

Catalase Test: positive.

Carbohydrates: Arabinose +; Xylose +; Glucose + Lactose +; Raffinose —; Glycerol +; Mannitol +; Sorbital +.

Litmus Milk Observation: Reaction: acidic; Acid curd: +; Rennet Curd: —; Peptonization: +; Reduction of Litmus: +; Gas Production: +.

Starch hydrolysis: —

Casein hydrolysis: +

KNO_3 reduced to KNO_2: +

Acetyl methyl carbinol produced: +

Methyl Red test: +

All the above tests on morphology and physiology of the isolate organisms seemed to agree with *M. caseolyticus* (23). However, from biosynthesis ex-

periments of glutamate it was found that the strains isolated differed from *M. caseolyticus*.

DISCUSSION

In various biological systems, alpha-ketoglutaric acid is taken to be the immediate precursor of L-glutamic acid (1). The synthesis of L-glutamic acid is thought to be either by L-glutamic dehydrogenase or by transaminase.

1. Glutamic dehydrogenase or reductive amination.

$$\text{alpha-ketoglutaric acid} + \text{TPNH} + H^+ + NH_3 = \text{glutamate} + \text{TPN} + H_2O$$

2. Transaminase.

$$\text{alpha-ketoglutarate} + \text{amino acids} = \text{glutamate} + \text{acids}$$

Glutamate is synthesized from alpha-ketoglutarate and ammonium chloride by a strain of *Pseudomonas ovalis* (5). Katagiri et al. also reported transaminase activity of *E. coli* (6). Both of these processes required alpha-ketoglutarate as the chief precursor for glutamate biosynthesis.

Since alpha-ketoglutarate is an intermediate of the TCA-cycle, the TCA-cycle is believed to be involved in the biosynthesis of glutamate. If the TCA-cycle is involved, the link between carbohydrate metabolism and the TCA-cycle is likely to be one of the members of the Embden-Meyerhof Scheme. At any event, the organism desired must be able to synthesize glutamic acid from carbohydrates and an amino group.

Whether alpha-ketoglutarate or other members of the TCA-cycle such as citrate may be the precursor of glutamate synthesis is also determined by this study. In this particular experiment attempts have been made to determine to what extent the TCA-cycle participates in glutamic acid biosynthesis. In this investigation, alpha-ketoglutarate is used only to study the mechanism of the TCA-cycle; it is not used as a precursor to glutamic acid biosynthesis. Carbohydrates were used as starting substrates.

Another possible glutamate synthesis pathway is by transamination. Since no other amino acids were added to the media used, the possibility of transamination is thus ruled out.

Glutamate biosynthesis by means of reductive amination is thought to be accomplished through two major pathways. The first pathway is the oxidation of the carbohydrates either by the Embden-Meyerhof Scheme or Hexose-monophosphate Shunt. The second pathway involves the reductive amination of alpha-ketoglutarate. The organism isolated and used in this study can perform glutamate synthesis in one major step aerobically by a pathway other than the TCA-cycle.

Glutamic acid could be easily obtained from degradation products of proteinaceous substances such as soybean meal, casein, and distiller's sol-

ubles by numerous microorganisms. However, this is a degradation product and is not really a microbial synthesized product. The success of this experiment lies in the fact that glutamate is synthesized from carbohydrates and amino group donor compounds. The present study has made extensive use of radiation from various radioactive sources with promising results.

According to the classical theories glucose is first oxidized to pyruvate anaerobically. From pyruvate we enter into the TCA-cycle. Here the TPN-specified dehydrogenase, glutamic dehydrogenase, is believed to be active and the presence of $-NH_4^+$ ions are absolutely necessary.

The pathway of glutamic acid biosynthesis described in this paper is seen not entirely to obey the TCA-cycle, as is evident in the presentation of the results. The exact pathway of glutamic acid biosynthesis pertaining to these experiments is not yet known.

The theory on which the entire study is based is to design a selective medium to isolate the desired organism from promising origins. The organism thus isolated is further subjected to radiation experiments with the hope that a mutant producing a high yield may be obtained. Radiation work is based on the theory of destruction, distortion and rearrangement of nuclear DNA. Irradiated cells have been observed to exhibit damages in DNP content and their DNA biosynthesis. The processes may be reversible but under higher doses of X-ray, injury is irreparable (12). Biochemical differences in mitochondria are also known to exist among mutant cells (13). The mutation work could be a long process because the G value for DNA is 0.0039 which is low in comparison with many other radiation sensitive protein molecules. G value is given as the number of molecules reacting per 100 ev (14).

Following irradiation, among the surviving colonies, biosynthetic capabilities are observed to vary. Some failed to produce glutamate, some will produce the usual amount, still others will produce a higher yield. Therefore screening of all survivals is necessary.

In theory, three steps are necessary for glutamate biosynthesis.

1. Glucose to pyruvate.
2. Pyruvate to alpha-ketoglutarate.
3. Alpha-ketoglutarate to glutamate.

It is the aim of this study to achieve a one-step biosynthesis from glucose to glutamate in a short time, avoiding the anaerobic step.

CONCLUSION

Judging from the experimental results, we are convinced that glutamate producing organisms can be successfully isolated from the soil by employing a properly designed selective medium, and the yield of glutamate can be

later improved by subjecting the isolated strains to radiation. X-ray and UV light were found to be equally effective.

The optimum fermentation conditions for the isolated strains are: pH—7, temperature 30°C-37°C, aerobic plus shaking, length of time—about 6 days, carbon source-glucose, and concentration—8% to 10%. Addition of 0.2% of yeast extract will enhance growth.

Under the optimum conditions the maximum yield of glutamate is estimated to be around 1 mg. per ml. Before the irradiation experiments, the original organisms isolated directly from soil produced glutamate only in the order of micrograms per ml. Radiation effects on biosynthetic ability varied. Among the population of the same colony, some cells had lost their synthetic ability of glutamate as detected from the broths, some were unaffected, and some acquired a higher rate of glutamate biosynthesis. These results could best be obtained through long processes of screening and testing.

The role of the TCA-cycle in glutamate biosynthesis has been much emphasized. Su and Yamada recently suggested the possibility of the cycle's mechanism in glutamate biosynthesis by microorganisms (20). Abelsen and Vogel have even confirmed by tracer technique that the TCA-cycle was actually involved in biosynthesis of amino acids (21). However, the strains we obtained by radiation are not able to convert citrate or alpha-ketoglutarate into glutamate in the presence of an amino group donor, either under aerobic or anaerobic conditions. When a cell free extract method was used, citrate and alpha-ketoglutarate still could not be converted into glutamate. Therefore, it seems that the TCA-cycle is not the only pathway involved in glutamate biosynthesis.

Glucose is in many cases a reliable substrate for glutamate biosynthesis but only when the amino group is present. It is safe to say that as far as the organisms used in this study are concerned, citrate and alpha-ketoglutarate are not the immediate precursors in the glutamate biosynthesis, yet it is also interesting to note that the irradiated organisms isolated could use both citrate and alpha-ketoglutarate as carbon sources for growth but glutamate was not synthesized. Perhaps this can be explained by the adaptation theory as has been reported by Halvorson and Spiegelman (22).

The most unusual phenomenon observed in the entire work was the behavior of these presumably heterotrophic strains in an autotrophic medium of TABLE 3. For instance A3-IV received a total of 810 r of X-ray and 75 minutes of UV light radiation and in the autotrophic medium made up of inorganic ions at pH 7 the strains could survive as long as 30 days. On the other hand, no sign of survival was detected from overnight suspension of cells in distilled water. *M. caseolyticus* (ATCC) did not survive in the autotrophic medium at all. The writer feels that the organism isolated in this

work could be a new strain, if not a new species, since it definitely differs from *M. caseolyticus* (ATCC) as far as metabolic pathway is concerned. Both the autotrophic test and the glutamate biosynthesis ability are strong evidences to support this belief.

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