TERMINAL REACTIONS IN TRYPTOPHAN BIOSYNTHESIS IN YEAST

by

PRISCILLA PRINCE SAUNDERS

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APPROVED:

Redacted for Privacy

Assistant Professor of Department of Microbiology

In Charge of Major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Chairman of School Graduate Committee

Redacted for Privacy

Dean of Graduate School

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Typed by Ruth Baines

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INTRODUCTION

Tryptophan is important in cell physiology not only as a component for protein synthesis but for its metabolic products which play an active role in cellular control. In animals 5-hydroxytryptamine, the plant auxins, and the ubiquitously distributed nicotinic acid in pyridine nucleotide coenzymes all serve as key regulatory components in physiological control.

The mechanism of tryptophan biosynthesis has been intensely studied in recent years. Several reports suggested that in yeast a unique series of reactions was involved. It was of comparative biochemical significance, then to undertake a more definitive analysis of the terminal reactions in tryptophan formation in an attempt to resolve the apparent discrepancies in the pathways reported in the literature.

In order to study the biosynthesis of tryptophan in Saccharomyces cerevisiae, a series of yeast mutants was selected. Analysis on metabolic intermediates accumulated by these auxotrophs as well as enzymatic studies were performed. The work was directed primarily towards a study of the conversion of anthranilic acid to

tryptophan.

For the sake of convenience, the problem reported herein has been grouped into three separate phases: (1) the characterization of mutants, (2) the conversion of anthranilic acid to indole-3-glycerol phosphate, and (3) the effect of glucose on the conversion of anthranilic acid to indole compounds.

HISTORICAL

Present knowledge of the biosynthetic pathway of tryptophan has come about through the efforts of investigators applying the principals of biochemical genetics. Although considerable study has been made on the intermediate reactions in this process, there are many questions that remain to be answered.

The first demonstration of an immediate precursor of tryptophan was published in 1940 by Fildes (8, p. 315-319) who was able to show that certain bacteria requiring trytophan for growth could utilize indole to satisfy this requirement. He went on to reason that ammonia was converted to tryptophan via indole.

In 1943, Snell (20, p. 389-394) showed anthranilic acid as a precursor of tryptophan when he discovered that certain species of Lactobacillus could use either anthranilic acid or indole to replace tryptophan. The sequence of these two compounds on the pathway was suggested by Tatum and Bonner (22, p. 349) when they found that Neurospora could convert indole plus serine to tryptophan.

Beadle and Tatum, (1, p. 499-506), in 1941, developed methods of isolating and characterizing mutants of Neurospora crassa. These techniques proved to be valuable research tools in the investigation of biosynthetic

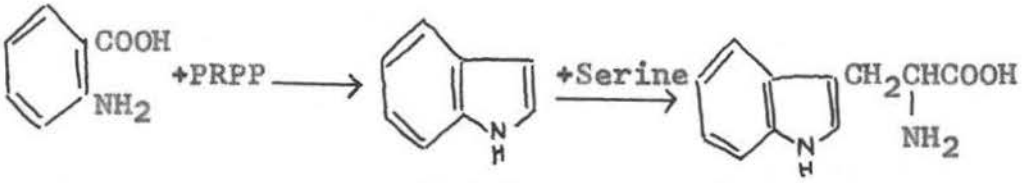
pathways. Using such methods, Tatum, Bonner, and Beadle (23, p. 477-478) found it possible to isolate various strains of Neurospora that were unable to synthesize tryptophan. By accumulation studies and feeding experiments they were then able to show that while the tryptophan requirement in one mutant could be satisfied by indole, in another it could be satisfied by either indole or anthranilic acid. This then suggested that anthranilic acid was a precursor of indole.

In 1946, Umbreit, Wood and Gunsalus (24, p. 731-732) demonstrated a requirement for pyridoxal phosphate in the conversion of indole plus serine to tryptophan by a cell free system. This was the first demonstration of a co-factor requirement in the system. Gordon and Mitchell (11, p. 110) named the enzyme catalyzing this conversion "tryptophan desmolase", which has been more recently changed to "tryptophan synthetase".

Nyc, et al. (16, p. 783-787), using radioactive tracer techniques, showed that the carboxyl carbon of anthranilic acid is not retained in tryptophan, while Partridge, Bonner, and Yanofsky (18, p. 269-278) demonstrated that the nitrogen atom is retained in the formation of the indole nucleus.

Yanofsky (26, p. 345-354) demonstrated, using E. coli, that the two non-benzenoid carbon atoms were derived from

glucose. He later showed (27, p. 594-595) that either ribose or ribose-5-phosphate served as excellent sources when used in conjunction with adenosine triphosphate (ATP), and that 5-phosphoribosyl-1-pyrophosphate (PRPP) could fulfill the requirement without the addition of ATP. Observations up to this point can be summarized as follows:



Anthranilic Acid Indole Tryptophan

In 1956, Yanofsky (28, p. 438-439), in a series of enzyme experiments, discovered indole-3-glycerol phosphate (IGP) as an intermediate between anthranilic acid and indole. Subsequently, Parks and Douglas (17, p. 207-208) published evidence suggesting the presence of a fructose derivative of anthranilic acid in the pathway preceeding indole in yeast. This would suggest that, in this system, anthranilic acid condenses with a hexose in place of a ribose as in E. coli and Neurospora. The compound corresponding to indole-3-glycerol phosphate would then be an indole-3-tetrose phosphate, unless there exists a mechanism for removal of the extra carbon atom.

Yanofsky and Rachmeler (31, p. 640-641) demonstrated in Neurospora that the conversion of IGP to indole and

and indole to tryptophan both occur on one enzyme. They showed that indole does not normally occur free, as it is bound to the enzyme. They suggested that the reaction proceeds as follows:

IGP + L-serine ⟶ L-tryptophan + triose phosphate

Crawford and Yanofsky (3, p. 1161-1170), in 1958, then showed that tryptophan synthetase is composed of two proteins, both of which are necessary for the aforementioned conversion. They designated the proteins A and B. Defective tryptophan synthetase having mutant protein A can convert indole to tryptophan, while a competent A and defective B effects the formation of indole from IGP. Both normal components are required for tryptophan synthesis from IGP.

Gibson, Doy, and Segall (10, p. 549-550) isolated the dephosphorylated form of an anthranilate derivative and suggested it as a possible intermediate in the biosynthesis of tryptophan. Smith and Yanofsky (19, p. 251-258) then identified a new intermediate preceeding IGP as 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CDRP). Recently, Gibson and Yanofsky (11, p. 489-500) have reported on the partial purification of IGP synthetase, and Doy, Rivera, and Srinivasan (7, p. 83-88) have published evidence for the enzymatic synthesis of N-(5'phosphoribosyl) anthranilic acid (PRA).

There have been no reports in the literature concerning an inhibitory effect of glucose on "tryptophan synthetase", however, Freundlich and Lichstein (9, p. 633-638) have demonstrated such an effect on tryptophanase.

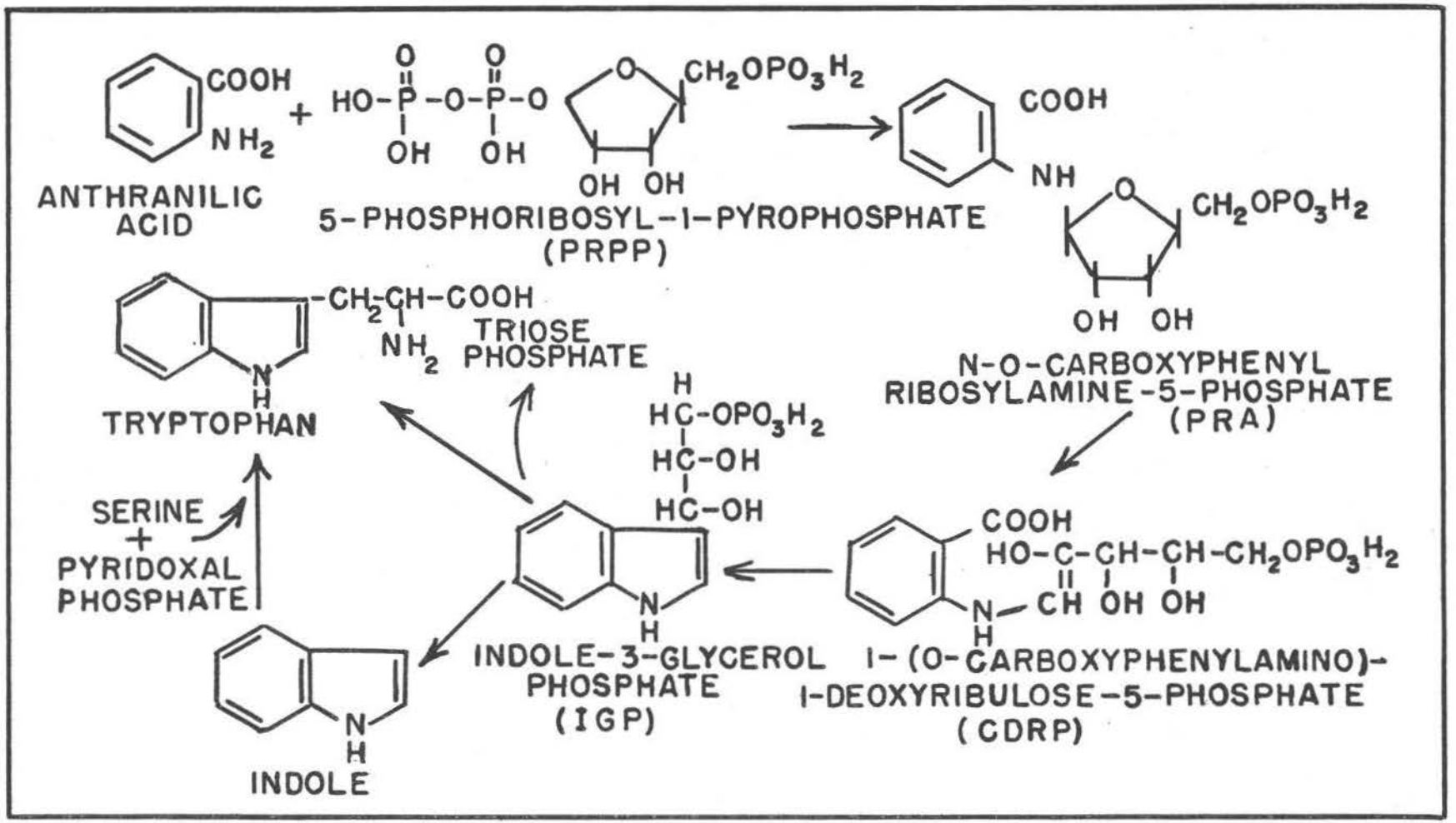


Figure I. Proposed pathway for the biosynthesis of tryptophan.

PART I

CHARACTERIZATION OF MUTANTS

In order to employ mutant organisms in the investigation of biosynthetic pathways, it is first necessary to obtain mutant strains requiring the compound in question. A characterization study is then carried out. The auxotrophs are screened by means of feeding experiments, detection of accumulating metabolic intermediates in the culture medium, and by enzymatic analysis to determine the biochemical lesions. By determining which metabolites the organism is unable to utilize and those that satisfy the requirement, it becomes a relatively simple matter to determine the general area of the biosynthetic pathway in which enzymes are missing or not functioning properly. In this study, the first two of the aforementioned characterization methods were employed prior to further analysis. It was felt that adequate preliminary information was obtained from these results.

Methods and Materials:

A modified Wickerham's medium (25, p. 293-301) was employed throughout this study and prepared according to the following formula:

Wickerham's Complete Medium

All Amounts Per Liter

H_3BO_3	0.01 mg	KH_2PO_4	0.875 g
$CuSO_4 \cdot 5H_2O$	0.01 mg	K_2HPO_4	0.125 g
KI	0.01 mg	$MgSO_4 \cdot 7H_2O$	0.50 g
$FeCl_3 \cdot 6\ H_2O$	0.05 mg	NaCl	0.10 g
$ZnSO_4 \cdot 7H_2O$	0.07 mg	$CaCl_2 \cdot H_2O$	0.10 g
Biotin	0.002 mg	Calcium pantothenate	0.4 mg
Thiamin·HCl	0.4 mg	Adenine sulfate	10.0 mg
Pyridoxine·HCl	0.4 mg	L-Histidine	5.0 mg
Uracil	10.0 mg	L-Methionine	5.0 mg
Inositol	2.0 mg	L-Tryptophan	5.0 mg

$(NH_4)_2SO_4$ 1.0 g

Glucose 20.0 g

The medium was prepared with limiting tryptophan (0.5 mg per liter in place of 5.0 mg) and distributed in the volumes of 100 ml per 250 ml flask. The flasks were inoculated and incubated on a rotary shaker at 30°C with ten ml aliquots being removed as desired for subsequent analysis. The cells were sedimented from aliquots of culture medium by centrifugation. Indole was extracted from a two ml aliquot with three ml of toluene, half of which, 1.5 ml, was saved for the colorimetric determination.

Indole-3-glycerol (IG) was assayed in the indole-free residue.

Indole was measured by its reaction with Ehrlich's reagent by the method of Stanley (21, p. 251-257). The reaction produced a red color, the absorbance of which was measured on a Beckman Model B Spectrophotometer at 570 mu wavelength.

IGP in the form of IG was measured by the method of Yanofsky (29, p. 171-184), which consists of the quantitative oxidation of IG to indole-3-aldehyde by sodium metaperiodate. The indole-3-aldehyde was then extracted with ethyl acetate and absorbance measured with a Beckman DU Spectrophotometer at 290 mu wavelength, assuming a molar extinction coefficient of 11,400.

Twenty microliters of each sample were then chromatographed in an ascending system, employing Whatman no. 1 paper. The solvent system used was n-butanol, n-propanol, water (1:2:1) made 0.005 N with respect to ammonium hydroxide. Solvent migration was allowed to proceed for 18 hours. Anthranilic acid was included as a reference compound on each sheet. After drying, the chromatograms were examined under ultraviolet light, since most of the compounds of major interest display strong fluorescence. Separated components were further revealed by spraying

the chromatograms with Ehrlich's reagent.

For the feeding experiments each mutant strain was grown for 48 hours in a culture tube containing ten ml of yeast complete medium (YCM), which is composed of 2 per cent glucose, 2 per cent tryptone, and 1 per cent yeast extract. The cells were harvested, washed twice with sterile distilled water, and resuspended to give a 1:100 dilution. One ml of this dilution was placed in a sterile petri plate to which was added 10 ml of melted Wickerhams complete medium lacking tryptophan. After sufficient agitation to get even dispersion of the yeast cells, the agar was allowed to solidify.

Sterile solutions of .001 M concentration of the following metabolites were tested:

1. Shikimic acid
2. Anthranilic acid
3. Phenylalanine
4. Indole
5. Tyrosine
6. Tryptophan

Sterile filter tabs were dipped into each solution and placed on the surface of the solidified agar in the Petri plate. These were then incubated for 24 hours, after which they were examined for areas of growth around the individual filter tabs. The presence of a zone of growth surrounding a filter tab was interpreted to mean that the metabolite contained in the tab could satisfy the mutant requirements for tryptophan. Wild type strain 1040-7C was included in each experiment as a positive control.

Results:

The feeding experiment revealed that three of the seven mutant strains were able to utilize indole as a means of satisfying the tryptophan requirement. The other four auxotrophs could not grow under these conditions, requiring exogenous tryptophan. The wild type, strain 1040-7c, could be cultured without supplementation for a tryptophan requirement.

Analysis of the culture medium (modified Wickerham's with limiting tryptophan) was carried out after 72 hours and again after 120 hours. IG appeared in every sample, however the range variation was pronounced (Table I). Four of the mutants, 4377, 4-5, 280, and 1071-3b accumulated far greater amounts than did the others. Following the accumulation of indole as well indicated that two of the aforementioned IG accumulators, 4377 and 4-5 also formed indole in quantities far greater than the rest of the mutants.

Paper chromatographic analysis of the culture medium was carried out after 72 hours and 120 hours. The data reported in Table II were obtained from the second analysis. After 72 hours two separated compounds were observed in each case, the one with the higher R_f value appearing to be identical to anthranilic acid. The second spot was at first assumed to be an accumulated intermediate

Table I

Growth requirements and accumulations by S. cerevisiae wild type and tryptophan auxotrophs

Strain	Compound Supplied*				Compounds Accumulated****			
	Shikimic Acid	Anthranilic Acid	Indole	Tryptophan	IGP 72 hrs	IGP 120 hrs	Indole 72 hrs	Indole 120 hrs
10713b	-**	-	+***	+	10.7	20.2	0.7	1.5
11692c	-	-	+	+	4.85	7.5	1.2	1.5
4-5	-	-	-	+	29.8	30.5	3.0	4.5
280	-	-	-	+	17.6	21.5	0.6	0.6
3727a	-	-	+	+	2.85	9.2	0.5	0.6
4377	-	-	-	+	30.1	39.2	3.0	4.0
10407c	+	+	+	+	4.85	10.5	0	0

*Filter feeding technique
**- = no growth
***+ = growth in 24 hours
****Compounds accumulated expressed in mμM per ml of culture medium.

Table II

Paper chromatographic analysis of culture medium of tryptophan auxotrophs of *S. cerevisiae*

Strain	R_f	Fluorescence*	Color Reaction with Ehrlich's reagent
1071 3b	0.53	+	Yellow
1169 2c	0.52	+	"
	0.36	+	"
	0.32	+	"
4-5	0.53	+	"
	0.39	+	"
	0.34	+	"
280	0.52	+	"
	0.36	+	"
	0.32	+	"
3727a	0.53	+	"
4377	0.56	+	"
	0.36	+	"
	0.32	+	"
1040 7c	-	-	-
Reference Compounds			
Anthranilic Acid	.52	+	Yellow
Anthranilic Acid+Glucose	0.52	+	"
	0.38	+	"

*Fluorescence determined by observation under a mineralite model SL 2537 ultra-violet lamp.

in the tryptophan biosynthetic pathway previously described by Parks and Douglas (17, p. 207-208); however, it was soon observed that a similar second spot appeared when anthranilic acid was chromatographed in the presence of glucose. After 220 hours a third compound appeared in four (1169 2c, 4-5, 280 and 4377) of the samples, while only one spot, that corresponding to anthranilic acid, appeared in the others. The third compound to appear possessed an R_f value slightly greater than the second spot observed on the 72 hour chromatogram.

The determination of enzyme activity in cell free preparations of the mutant strains was somewhat hampered by the crudeness of the preparations and the fact that the cells were originally grown in a very complete medium (YCM). Activity was observed in the controls; however, it was possible to estimate a general degree of produce accumulation which was assumed to be sufficient for preliminary mutant characterization. For the sake of simplification the data are reported in this manner in Table III. Four of the preparations displayed a relatively high degree of activity, those derived from strains 1071-3b, 4-5, 280, and 1070 7c. The rest were not considered significant.

Discussion:

According to the nutritional and accumulation data presented here, the six tryptophan auxotrophs studied fall

Table III

Accumulation of IGP by crude cell free preparations* of tryptophan auxotrophs of S. cerevisiae

	muM of IGP produced per mg protein in 1 hour		
Strain	Complete**	Without Anthranilic acid	Without R-5-P
1071 3b	8.0	2.0	2.0
1169 2c	5.0	3.0	3.0
4-5	4.0	1.0	2.5
280	16.0	4.0	7.0
3727 a	1.9	1.7	1.6
4377	4.0	1.6	3.0
1040 7c	1.8	0.5	0.6

*Methods concerning cell free preparations are contained Part III. Cells were grown in YCM, harvested, and aerated as described.

**Complete reaction mixture as described in Table IV.

into five classifications:

1. Mutant strain 3727a appears to possess a genetic deficiency impairing the condensation of anthranilic acid with PRPP, assuming the reaction sequence to be similar to that in E. coli as described by Smith and Yanofsky (19, p. 251-258). The single compound appearing on the 120 hour chromatogram was analogous to anthranilic acid and would suggest such a block.
2. 1169-2c is assumed to be genetically unable to form indole-3-glycerol phosphate from its immediate precursor, CDRP. The small amounts of indole accumulated in the culture medium could possibly have resulted from the breakdown of a small amount of tryptophan by the enzyme tryptophanase yielding indole. The IG present could then be explained by the reversibility of the IGP $\longrightarrow$ indole reaction.
3. Strain 1071-3b accumulated IG, but could effect the conversion of indole $\longrightarrow$ tryptophan. The genetic lesion here would seem to be manifesting itself in the form of an altered A protein that has retained the ability to combine with the B protein. The latter would thus be able to catalyze the conversion of indole to tryptophan

unhampered.

4. With the exception of the nutritional studies, strain 280 exhibited characteristics similar to those of 1071 3b. They both accumulated IG and could not effect the conversion of IG to indole. Strain 280, however, could not have its tryptophan requirement satisfied by indole. These data strongly suggest either of two possibilities:
    a. Both the A and B proteins are altered as the result of different genetic lesions.
    b. There is one mutated site affecting the binding of the two proteins, thus rendering them both virtually inactive.
5. In view of the fact that strains 4-5 and 4377 accumulated large amounts of IG and somewhat lesser amounts of indole, it is proposed here that these two auxotrophs nurture similar protein alterations. The defect is suspected to be associated with the requirement for pyridoxal phosphate since the two reactions affected are those requiring this cofactor. The accumulation of such small amounts of indole can perhaps be explained by the slow rate at which this reaction proceeds. Yanofsky (30, p. 225) has shown that

the rate of this reaction in either direction is less than 5 per cent the rate of the other two reactions associated with tryptophan synthetase.

The location of the discussed metabolic blocks in the biosynthetic pathway are illustrated in Figure II.

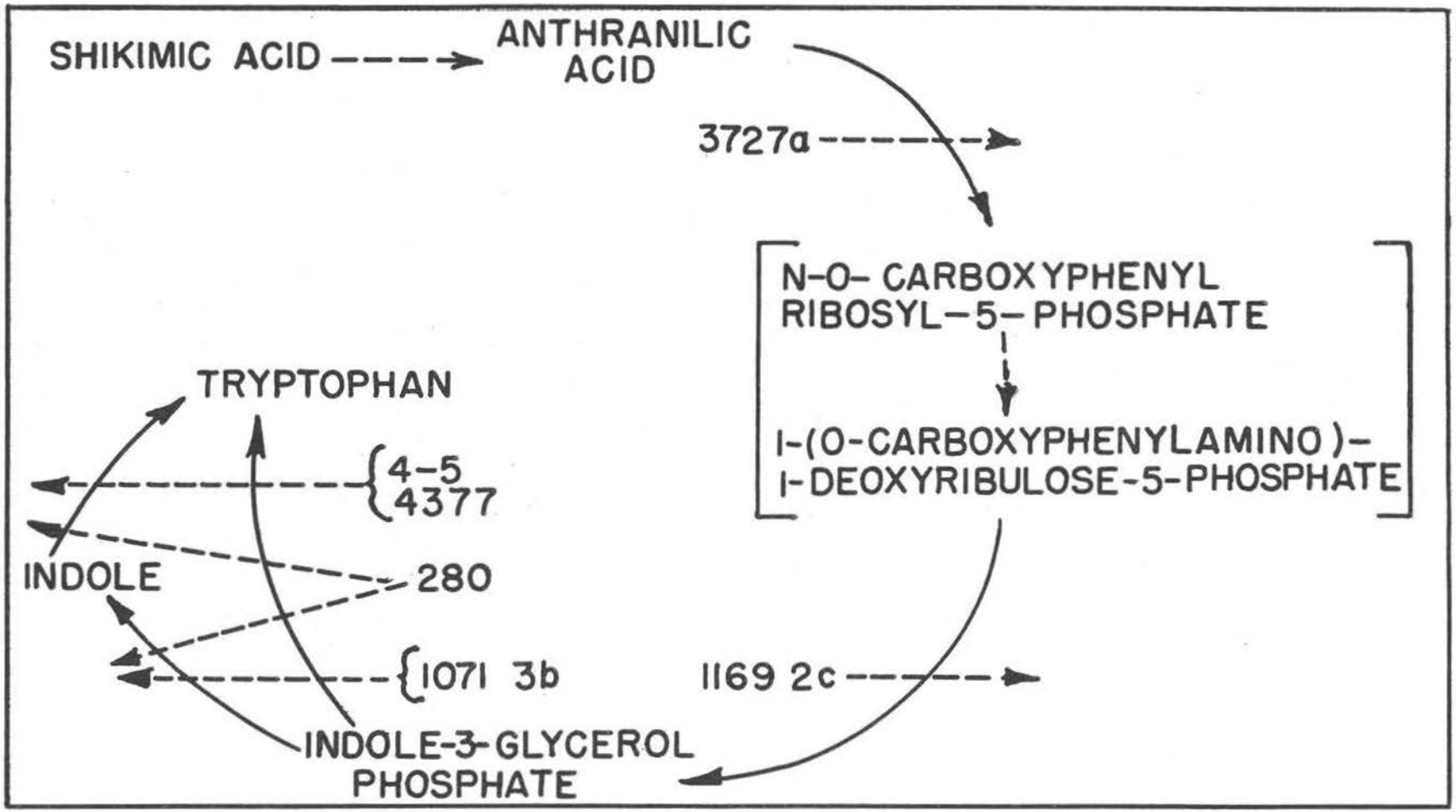


Figure II. Proposed mechanism of tryptophan biosynthesis with suspected sites of enzymatic deficiencies in mutant strains studied.

Part II

THE CONVERSION OF ANTHRANILIC ACID TO INDOLE-3-GLYCEROL PHOSPHATE

The enzymatic conversion of anthranilic acid to indole-3-glycerol phosphate (IGP) has been studied in great detail in Escherichia coli and Neurospora crassa primarily by Yanofsky and his coworkers. In these systems it is clear that anthranilic acid condenses with a ribose derivative, 5-phosphoribosyl- pyrophosphate (PRPP), as shown by Yanofsky (27, p. 594-595). In yeast and Salmonella typhimurium, however, there appears to be some confusion as to whether the initial step in this reaction sequence involves a ribose derivative, as in E. coli, or rather a hexose. The latter is suggested through reports by Parks and Douglas (17, p. 207-208) and Lingens, et al. (13, p. 727-729) concerning the accumulation of fructose derivatives of anthranilic acid in the culture medium of tryptophan auxotrophs of Saccharomyces cerevisiae and S. typhimurium respectively. The following study was initiated in an attempt to clarify this problem after working out methods of obtaining cell-free systems of relatively high activity.

It has been demonstrated by Doy and Pittard (6, p. 941-942) that tryptophan can exert feedback control over the formation of anthranilic acid, and Moyed and Friedman (15, p. 968-969) have suggested a similar inhibition of the condensation of anthranilic acid with PRPP. In view of this information, it was reasoned that in order to obtain maximum activity in a cell-free preparation, a defined synthetic medium should be employed as a means of limiting the available trytophan. This was accomplished using a modified Wickerham's medium as outlined in a previous section of this study.

In cases where maximum possible activity was not necessary, the cells were grown in a complete medium (YCM), harvested, resuspended in 0.1 M KH_2PO_4 at pH 6.0, and supplemented with 100 μg per ml anthranilic acid. This was done in an attempt to induce higher levels of the enzymes concerned.

Experimental Methods:

Modified Wickerham's complete medium was prepared with limiting tryptophan in 10 liter quantities and placed in 5 gallon pyrex bottles. A cotton stopper was prepared, through which passed a frittered glass aeration tube which extended to the bottom of the bottle. An air filter consisting of a 5 inch pyrex tube packed with sterile cotton was attached to the opposite end of the aeration tube.

The complete apparatus and media were sterilized by autoclaving at 135°C for one hour. A 250 ml flask, containing 100 ml of YCM was inoculated and incubated on a rotary shaker at 30°C for 12 to 14 hours. Twenty to 30 ml of this culture were then used as an inoculum for the 10 liters of Wickerham's medium. The cells were grown with vigorous aeration for 24 to 48 hours at room temperature, after which they were harvested with a Serval SS-3 centrifuge equipped with a "Szent-Gyorgyi and Blum" 8-tube Continuous Flow System. The cells were then washed twice with distilled water and resuspended in cold 0.1 phosphate buffer at pH 7.8 to a concentration of approximately 55 mg dry weight per ml. The cells were disrupted in a 10 kc Raytheon Sonic Oscillator and the cellular debris was removed by centrifugation at 100,000 x g for 30 minutes in a Spinco Model L ultracentrifuge. The supernatant was then fractionated or immediately dialyzed against approximately 100 volumes of 0.05 M KH_2PO_4 at pH 7.8 for 3 hours with stirring at 4°C. The cell-free preparation was then stored at -20°C as a crude extract. Due to rapid loss of activity, the preparations were stored no longer than 2 days.

Results:

The study of the effect of aeration of cells in the

presence of anthranilic acid and glucose prior to sonic disruption revealed a notable stimulation of activity when limited to two hours. As illustrated in Table IV, prolonged aeration gave rise to a marked diminution of activity with relation to the conversion of anthranilic acid to IGP.

The results in Table V imply that ribose-5-phosphate (R-5-P) is condensed more readily with anthranilic acid than either of the hexose phosphates used. Of the latter, fructose-6-phosphate (F-6-P) imparted slightly more activity than did glucose-6-phosphate (G-6-P). Both the wild type, in the form of commercial yeast cake (Fleischman's) and mutant strain 4-5 yielded similar results, although the overall activity of the 4-5 preparation was somewhat less than the wild-type. As a follow up to this experiment, a similar one was conducted, employing R-5-P and G-6-P. The progress of the reaction was followed by measuring the accumulation of IGP. After a period of time triphosphopyridine nucleotide (TPN) was added to two of the reaction mixtures and, as shown in Figure III, IG synthesis increased significantly with G-6-P, while that with R-5-P decreased.

Discussion:

The demonstration of the increase in enzyme activity by aeration of cells prior to sonic disintegration can be

Table IV

The effect of aeration* on activity of crude cell free preparations of commercial yeast cake (Fleischman's)

Treatment of Cells	mμM IGP Formed/mg Protein/Hour***		
	Complete Reaction Mixture**	Without Anthranilic Acid	Without R-5-P
No Aeration	12.6	4.0	4.4
Aeration 2 Hours	60.8	9.0	14.0
Aeration 12 Hours	5.6	1.8	0.6

*Aeration in 0.1 M phosphate buffer at pH 7.0 supplemented with 100 μg per ml anthranilic acid and 1.0 per cent glucose.

**Complete reaction mixture was composed of the following:

0.002 M anthranilic acid	0.2 ml
0.1 M ribose-5-phosphate	0.1 ml
0.1 M ATP	0.1 ml
0.01 M $MgSO_4$	0.1 ml
cell-free preparation	1.0 ml

The mixture was made up, in each case, to 2.0 ml with 0.1 M phosphate buffer at pH 7.8.

***Protein was determined by the method of Lowry (14, p. 265-275)

Table V

Efficiency of sugar phosphates in the conversion of anthranilic acid to IGP in cell-free extracts of S. cerevisiae

Strain	Reaction Mixture	mμM IGP Formed/mg Protein/Hour		
		Sugar Phosphate		
		R-5-P*	G-6-P**	F-6-P***
Commercial yeast cake (Fleischman's)	Complete	50.2	17.2	21.6
	Without anthranilic acid	5.8	9.6	5.6
	Without sugar phosphate	7.2	8.4	7.2
4-5	Complete	15.0	3.6	5.4
	Without anthranilic acid	6.8	5.6	5.0
	Without sugar phosphate	5.4	5.0	4.6

*R-5-P = ribose-5-phosphate
**G-6-P = glucose-6-phosphate
***F-6-P = fructose-6-phosphate

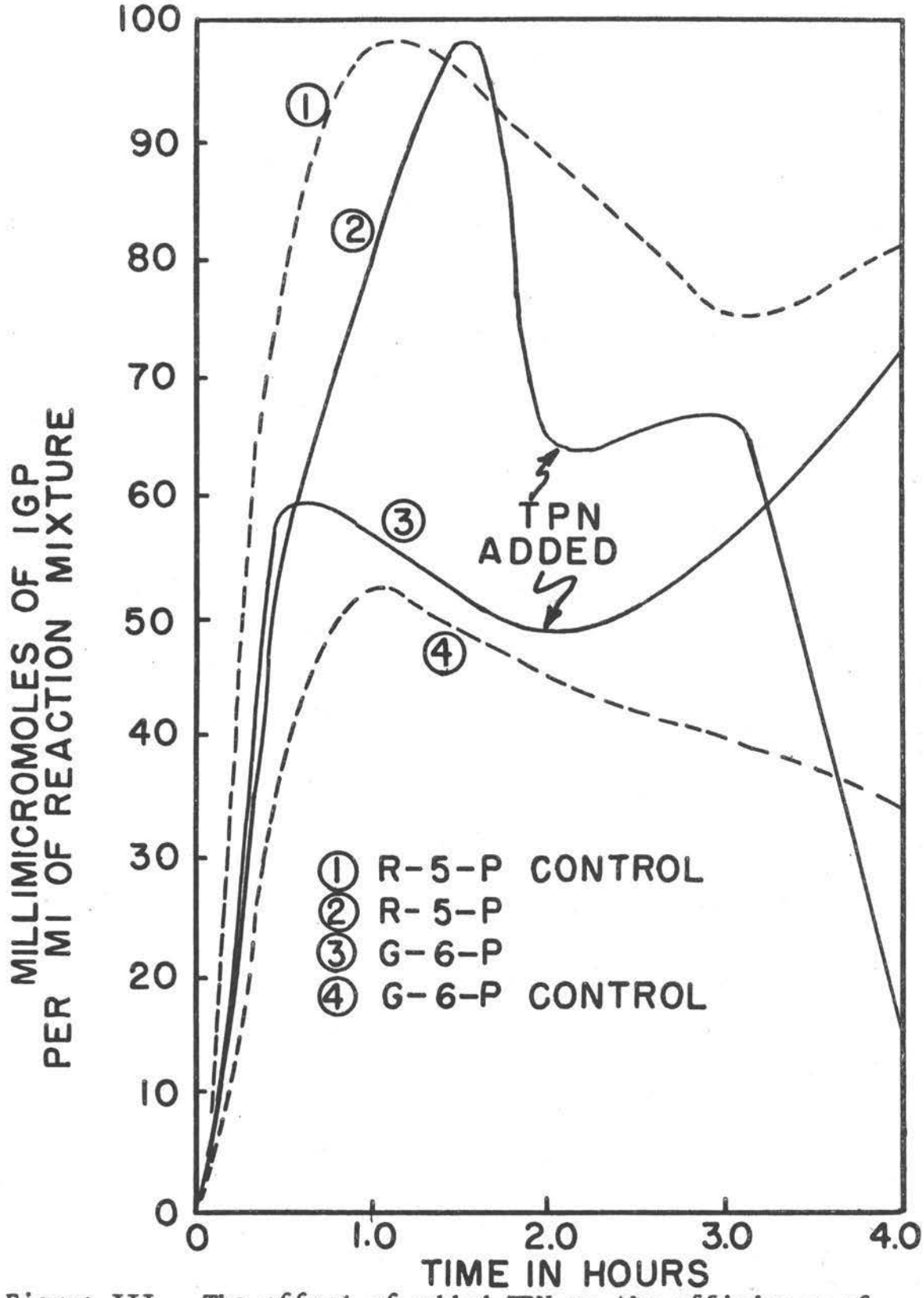


Figure III. The effect of added TPN on the efficiency of sugar phosphates with respect to IGP formation.

explained by several possibilities. First, it has been shown by Halvorson (12, p. 255-256) that enzyme synthesis can proceed in nongrowing yeast cells as a result of protein turnover. The presence of glucose has been shown (Part III) to stimulate the uptake and utilization of anthranilic acid. Assuming that the commercial yeast were grown in a rich medium (tryptophan present in excess), it can be proposed that the original enzyme level was quite low due to repression by the amino acid. It then follows that exposure of these washed cells to anthranilic acid under the stimulatory effect of glucose could relieve this repression such that the enzyme level could be increased. Prolonged incubation in this state could reverse the situation due to tryptophan accumulation.

The fact that R-5-P is more efficient in the conversion of anthranilic acid to IGP than hexose derivatives was not altogether expected; however, the results obtained seem quite conclusive. Since there was a lesser degree of activity with the hexoses, it was suspected that it was the result of their conversion to R-5-P. As the cell free preparations were crude, it seemed logical that there could have been sufficient TPN present to effect a slight degree of activity. This hypothesis was tested and appeared to be confirmed as illustrated in Figure III. The increase in the accumulation of IGP with G-6-P can be

attributed to the following reaction sequence of the pentose cycle:

(1) G-6-P + TPN^+ ⟶ 6-phosphogluconate + TPNH + H+

(2) 6-phosphogluconate + TPN^+ ⟶ ribulose-5-phosphate + TPNH + H^+

(3) Ribulose-5-phosphate ⟶ R-5-P

The activity obtained previously with F-6-P would have entered the same pathway after its direct conversion to G-6-P. From these data it appears that this part of the biosynthetic pathway of tryptophan is identical with that of E. coli and Neurospora.

Part III

THE EFFECT OF GLUCOSE ON THE CONVERSION OF ANTHRANILIC ACID TO INDOLE COMPOUNDS

There have been several reports of the inhibitory effect of glucose on the formation of certain inducible enzymes in microbial systems. Recently such an effect was observed on the formation of the tryptophanase enzyme in E. coli by Freundlich and Lichstein (9, p. 633-638). Some data obtained in this laboratory were suggestive of a glucose effect on indole formation in yeast.

The following experiments constitute preliminary efforts to design an experimental system whereby the effect of glucose on the conversion of anthranilic acid to indole compounds could be studied in non-proliferating cells of S. cerevisiae. After growing the cells in YCM for 48 hours, glucose is exhausted from the medium. The levels of the tryptophan synthesizing enzymes were quite low because of inhibition of enzyme synthesis and/or activity by the presence of tryptophan in the intracellular environment. Such feedback control has been observed in the tryptophan biosynthetic pathway in E. coli by several investigators (6, p. 941-942 and 15, p. 968-969). It is suggested by Doy (4, p. 185-205) that both inhibition of enzyme synthesis and activity are involved.

Experimental Methods:

The following solutions were prepared for use in this series of experiments:

1. 1.0 mg per ml anthranilic acid with pH adjusted to 7.0 with NaOH
2. 20 per cent glucose
3. 0.5 M and 1.0 M hydroxylamine hydrochloride with pH adjusted to 7.0 with NaOH
4. 0.1 M KH_2PO_4 at pH 7.0

These solutions were dispensed into 250 ml flasks as prescribed in each experiment such that every flask contained 25 ml of the incubation mixture. Each experiment included a control flask on anthranilic acid, glucose, and hydroxylamine hydrochloride.

The cells were grown in 1 liter of YCM contained in a 2 liter Erlenmeyer flask on a rotary shaker at 30°C. After 48 hours growth the cells were harvested by centrifugation and washed twice with distilled water. The packed cells were then resuspended in 0.1 M KH_2PO_4 at pH 7.0 such that a 1 to 50 dilution gave an O.D. at 525 mμ wavelength of approximately 0.5.

At the onset of the experiment the flasks containing the incubation mixtures were placed in crushed ice and 25 ml of the cell suspension were added to each. After sufficient mixing 6.0 ml aliquots were immediately removed, centrifuged to remove the cells, and the

supernatant saved for analysis. The flasks were then placed on a rotary shaker at 30°C with 6 ml aliquots being removed as desired, centrifuged, and the supernatant retained for analysis.

Indole and IGP were measured as described above and on (page 11). For the determination of anthranilic acid, one ml samples of the culture medium were dispensed in tubes and made up to 10 ml with 0.1 M KH_2PO_4 at pH 6.0. The characteristic fluorescence of the compound was then measured with an Aminco Bowman Spectrophotofluorometer, employing an activation wavelength of 315 mu and measuring the fluorescence at 415 mu.

Results:

The first experiment in this series was designed to determine the optimum concentration of hydroxylamine hydrochloride for preventing the further utilization of both IGP and indole. Constant amounts of anthranilic acid, 0.5 μM per ml, and glucose, 2.0 per cent, were included in each incubation flask with varied concentrations of hydroxylamine. Samples were assayed periodically for IG and indole. The greatest accumulation of indole occurred in the presence of 0.05 hydroxylamine, while IG appeared in two peaks with respect to hydroxylamine concentration (Figure IV). The lesser of the two peaks occurred under the influence of 0.025 M hydroxylamine, very close to the peak of indole accumulation. Concentrations greater than 0.3 M hydroxylamine resulted in the greatest accumulation of IG, while indole production increased only slightly.

The effect of glucose on the system was determined in a preliminary experiment, the results of which are described in Table VI. From the data recorded here it is quite clear that the uptake of anthranilic acid is strongly stimulated by increasing amounts of glucose.

The greatest stimulation of IG production at 2 hours was found in the presence of 0.5 per cent glucose. For longer incubation periods the greatest change occurred

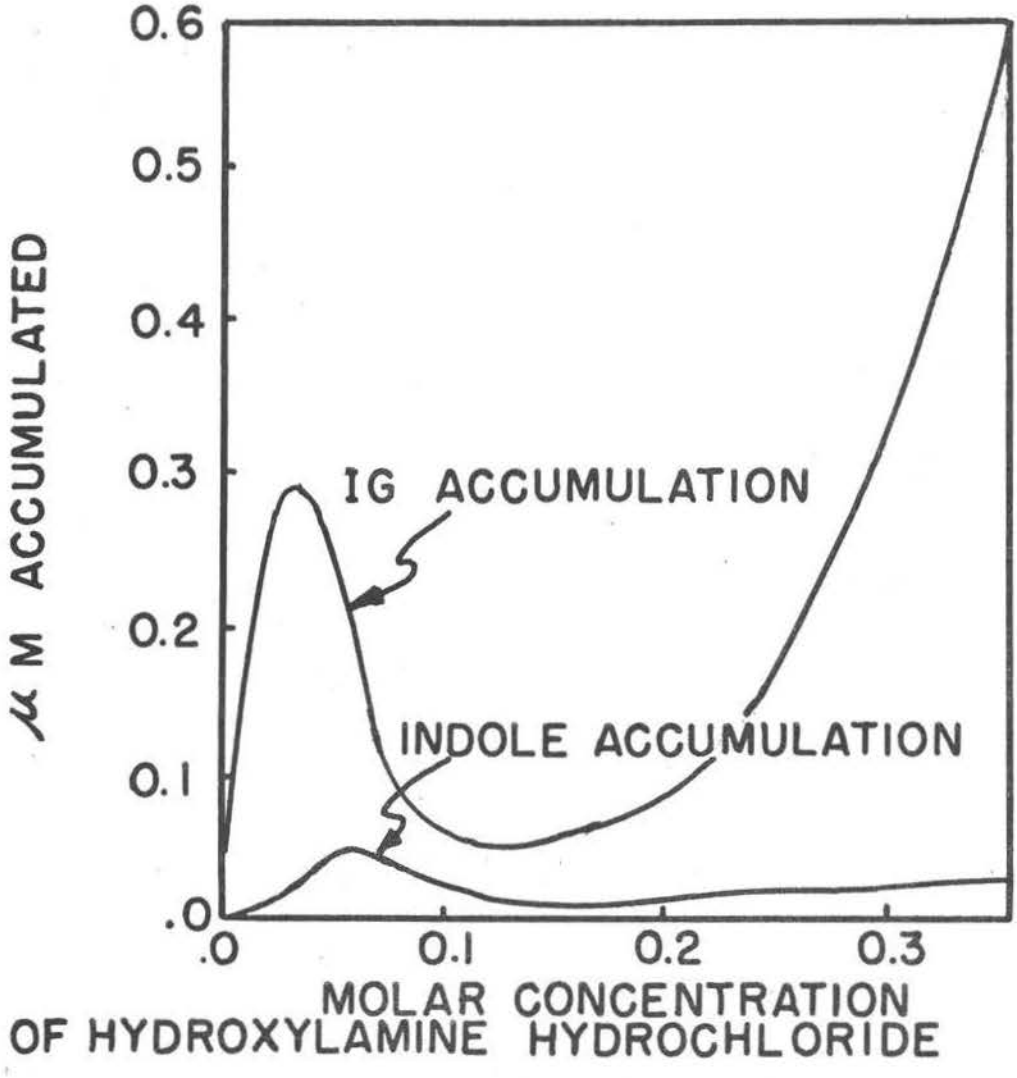


Figure IV. The effect of hydroxylamine on the accumulation of IG and indole by whole cells of Saccharomyces.

Table VI

The effect of glucose on the uptake of anthranilic acid and the accumulation of IG and indole in S. cerevisiae

	0 Hours			2 Hours			4 Hours			6 Hours		
% Glucose	Change in: AA**	IG*	Indole*	AA	IG	Indole	AA	IG	Indole	AA	IG	Indole
0	320	0	0	340	28	2	350	21	0	355	12	6
4.0	210	0	0	160	34	15	110	52	18	50	60	37
2.0	220	0	0	180	44	16	160	72	28	160	68	27
1.0	260	0	0	200	57	9	230	161	33	200	145	37
0.5	290	0	0	250	172	5	270	77	20	230	29	44
0.25	310	0	0	310	164	5	290	88	19	260	100	18

*Reported as the change in mμM per ml of incubation mixture.
**Anthranilic acid is reported as mμM remaining per ml of incubation mixture.

with 1.0 per cent glucose supplementation.

At 2 hours the most distinctive increase in the accumulation of indole occurred in the incubation mixture containing 2.0 per cent glucose, while at 4 hours the greatest change was found with 1.0 per cent glucose. Another shift of stimulation was found from 4 to 6 hours, during which time there was a greater accumulation of indole in the incubation mixture containing 0.5 per cent glucose.

Discussion:

The inclusion of hydroxylamine as an inhibitor of tryptophan synthetase was stimulated by the earlier discovery that pyridoxal phosphate was an essential cofactor for the tryptophan synthetase reactions. The optimum concentration of hydroxylamine for purposes described herein was at first assumed to be that concentration at which the greatest accumulation of indole resulted, .05 M. It was reasoned that concentrations above this, with which the accumulation of indole would decrease, would essentially be causing the inactivation of tryptophan synthetase by interfering with the binding of the two protein units of the enzyme. This would be accomplished through the complete inhibition of pyridoxal phosphate.

The peculiar accumulation of IG with increasing amounts of hydroxylamine was not as expected. Concentrations of

inhibitor between 0.1 and 0.2 M appeared to be virtually ineffective, while less than 0.1 M and greater than 0.2 M both proved to be extremely effective with respect to IG accumulation. This phenomenon could possibly be explained by the existance of hydroxylamine reductase in yeast, if it were such that it required a concentration of hydroxylamine greater than 0.05 M for stimulation. An incubation mixture somewhat greater than 0.2 M with respect to hydroxylamine could possibly result in saturation of the enzyme such that excess inhibitor could again be effective. Preliminary experiments are being carried out in an effort to determine the presence of such an enzyme, but the results to this point have been inconclusive.

Repression of enzyme synthesis by glucose in the tryptophan biosynthetic pathway was suggested by the data concerning indole accumulated. The rate of indole formation appeared to be inhibited somewhat by increasing concentrations of glucose. On closer analysis, however, it was observed that the combined yield of IG and indole could not account for the amount of anthranilic acid that had disappeared from the incubation mixture. It is suggested here that, due to the accumulation of substrate resulting from glucose stimulation of the uptake of anthranilic acid, the condensation of hydroxylamine with pyridoxal phosphate became reversed. Inhibition by hydroxylamine has been

shown to be reversed by an excess of substrate or coenzyme (2, p. 150). This could account for the reactivation of tryptophan synthetase and the disappearance of indole.

SUMMARY

Nutritional and accumulation studies of a group of tryptophan auxotrophs of S. cerevisiae blocked between anthranilic acid and tryptophan resulted in 5 distinct classifications. Three of the five types reflect genetic deficiencies affecting tryptophan synthetase. Of the other two, one is unable to effect the condensation of anthranilate with PRPP, while the other is unable to form IGP synthetase.

Enzymatic experiments revealed that R-5-P is converted more readily than hexoses to the compound, probably PRPP, that condenses with anthranilic acid. The addition of TPN was shown to enhance IGP formation from anthranilic acid and G-6-P, further suggesting that a ribose derivative condenses with anthranilic acid in the tryptophan biosynthetic pathway in yeast.

Inhibition studies of tryptophan synthetase by hydroxylamine suggest that this repression of activity is reversed by the accumulation of IG. The possibility of the presence of hydroxylamine reductase is also suggested. Conclusive results were not obtained regarding the effect of glucose on the conversion of anthranilic

acid to indole due to problems encountered in the inhibition of tryptophan synthetase.

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