THE PARTIAL PURIFICATION AND PROPERTIES OF INDOLE-3-GLYCEROL PHOSPHATE SYNTHETASE FROM *ESCHERICHIA COLI*

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(Received February 6th, 1960)

SUMMARY

The enzyme which converts 1-*o*-carboxyphenyl-1-deoxyribulose-5-phosphate (anthranilic deoxyribulotide) to indole-3-glycerol phosphate has been partially purified from cell extracts of *Escherichia coli* and some of its properties examined. By growing tryptophan auxotrophs on media in which the available tryptophan was limited, crude extracts were obtained with up to 60 times the specific activity of similar extracts from wild type *E. coli*. A further 17-fold increase in specific activity was achieved by acetone precipitation followed by ammonium sulphate fractionation.

Experiments employing the mixing of fractions obtained during purification and the mixing of crude extracts of a number of tryptophan auxotrophs of *E. coli* strongly suggest that the conversion of anthranilic deoxyribulotide to indole-3-glycerol phosphate is carried out by a single enzyme.

A number of compounds with structural similarities to the substrate were tested as possible inhibitors of indole-3-glycerol phosphate synthetase. Anthranilic acid and certain of its derivatives were found to inhibit the enzyme.

INTRODUCTION

A number of tryptophan auxotrophs of *Escherichia coli*, *Aerobacter aerogenes* and *Salmonella typhimurium* have been found to accumulate 1-*o*-carboxyphenylamino-1-deoxyribulose[1,2]. Cell-free extracts of such strains convert anthranilic acid to 1-*o*-carboxyphenylamino-1-deoxyribulose-5-phosphate (anthranilic deoxyribulotide) but not to indoleglycerol phosphate, while extracts from other tryptophan auxotrophs contain an enzyme (or enzymes) which convert anthranilic deoxyribulotide to InGP[2].

Abbreviations: InGP, indole-3-glycerol phosphate; DEAE, diethyl-aminoethyl; Tris, tris-(hydroxymethyl)aminomethane.

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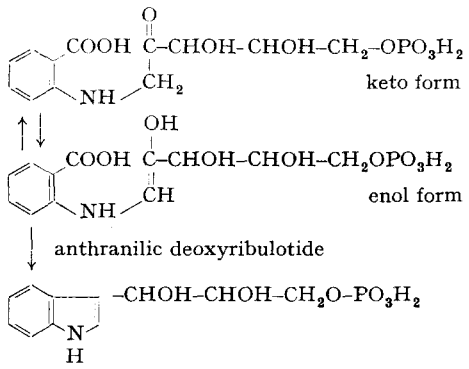


indole-3-glycerol phosphate

The object of the present communication is to report on the partial purification of this enzyme, for which the name indole-3-glycerol phosphate synthetase (InGP synthetase) is proposed, and on some of its properties. As the overall reaction involves the removal of both CO_2 and H_2O and ring closure, it was of particular interest to examine whether one or more enzymes were involved in the conversion.

MATERIALS AND METHODS

Organisms

A number of tryptophan auxotrophs of *E. coli* were employed in this study. In most experiments *E. coli* T-8, described by YANOFSKY[3] was used. This strain and the 14 strains of *E. coli* employed in the extract-mixing experiments were all derived from *E. coli* K-12. The wild type *E. coli* 518 and the tryptophan auxotroph (7-4) derived from it have been described by GIBSON, JONES AND TELTSCHER[4]. All the auxotrophs used were obtained by penicillin selection following u.v. irradiation.

Chemicals

All the chemicals used with the exception of anthranilic deoxyribulotide were obtained commercially and were not purified further. Chromatography on DEAE cellulose was carried out using "Selectacel", DEAE Reagent Grade, obtained from Brown Company, Berlin, New Hampshire, U.S.A.

The synthesis of anthranilic deoxyribulotide has been described[2]. Briefly the sodium salt of ribose-5-phosphate was heated with an excess of anthranilic acid in the presence of ethyl malonate. Excess anthranilic acid was then removed by three successive extractions with ethyl acetate and the solvent in the aqueous layer removed by gentle warming under vacuum. The resulting aqueous solution was kept at $-15°$ and used as the stock solution of anthranilic deoxyribulotide. The yield of anthranilic deoxyribulotide, as assayed by the amount of InGP formed by InGP synthetase was about 10 % of theoretical based on the amount of ribose-5-phosphate in the reaction mixture. Although a large excess of ribose-5-phosphate was presumably present in the solution of anthranilic deoxyribulotide it appeared to have no inhibitory effects on the reaction studied.

Buffers: These were Tris–HCl mixtures and mixtures of potassium phosphates.

Estimation of InGP synthetase activity

The incubation mixture for studying the conversion of anthranilic deoxyribulotide to InGP contained the following in a final volume of 0.5 ml. Anthranilic deoxyribulotide (0.22 μmoles), Tris buffer (pH 8.8, 25 μmoles) and enzyme solution made up to the final volume with distilled water and any other additions. Tests were incubated for 20 min at 37°.

The reaction was stopped by the addition of 0.2 ml of *M* acetate buffer (pH 5). This was followed immediately by 0.5 ml of 0.2 *M* aqueous sodium meta periodate to quantitatively convert the InGP formed to indole-3-aldehyde[5]. The mixture was allowed to stand at room temperature for 20 min after which 0.4 ml of *N* NaOH and 5 ml of ethyl acetate were added. After shaking 10–20 times to extract the indole-3-aldehyde the tubes were centrifuged at low speed for a few minutes and the O.D. at 290 mμ of the ethyl acetate layers measured against an ethyl acetate blank. The extinction coefficient of indole-3-aldehyde at 290 mμ is 11,400.

The unit of enzyme activity

One unit of enzyme activity is taken as that amount of enzyme required to form 0.1 μmole of InGP in 20 min at 37°. The use of this unit enables direct comparison with the next sequentially related enzyme, tryptophan synthetase. Specific activities are expressed as the units of enzyme activity /mg of protein. Protein was determined with FOLIN's phenol reagent[6].

Preparation of cell extracts

The cells were grown on the citric acid–mineral salts mixture described by VOGEL AND BONNER[7] supplemented with 0.16% glucose and 0.005% acid hydrolysed casein (neutralized before use). Unless otherwise stated 2 μg/ml of indole was included in the medium for the tryptophan auxotrophs.

For small batches, 1-l quantities of medium were inoculated with a 6–8 h culture in nutrient broth to give an initial cell population of about 10^6/ml. The cultures were then vigorously shaken for 16 h at 37°. For a large batch, 15 flasks each containing 1 l were prepared as above and used as inoculum for 45 l of fresh warm medium in a stainless steel tank and vigorously aerated at 37° for 7.5 h.

The grown cultures were harvested using a Sharples super centrifuge and the cells washed once with cold 0.9% NaCl. After resuspending in 0.1 *M* Tris buffer, pH 7.8 to give a final cell concentration of about $4 \cdot 10^{11}$ cells/ml the cells were disrupted for 20 min in a 10 kc Raytheon sonic oscillator. Cell debris was removed by centrifuging at 144,000 × *g* in a Spinco Model L centrifuge for 30 min. The supernatant fluid (crude extract) was stored at —15°. Such extracts had a protein content of about 20 mg/ml.

Enzyme preparations

Unless otherwise stated the enzyme preparation used was obtained by collecting the protein precipitated from a crude extract by ammonium sulphate fractionation (26 to 40%) The conditions under which such a precipitation was carried out are described in the section on purification.

RESULTS

Purification by fractionation

The most important factor in obtaining InGP synthetase preparations with a high specific activity was limiting the amount of tryptophan available for growth of tryptophan auxotrophs of *E. coli*. As can be seen from Table VII under these conditions crude extracts are obtained with a specific activity about 60 times as high as that of a corresponding extract made from wild type *E. coli*.

Attempts were made to purify the InGP synthetase from *E. coli* T-8 extracts by first removing nucleic acid and then using ammonium sulphate precipitation acetone precipitation, chromatography on DEAE cellulose, acid precipitation, heat, treatment and adsorption on calcium phosphate gel. The last 3 procedures have not as yet proven very useful and will not be described.

The most successful method of purification found so far is a combination of acetone and ammonium sulphate precipitations. As can be seen from the experiments summarized in Table I, it was most important to use the acetone precipitation as the first step. Attempts to follow ammonium sulphate fractionation with acetone resulted in poor recoveries and no significant purification.

TABLE I

FRACTIONATION OF STREPTOMYCIN-TREATED EXTRACT WITH ACETONE AND AMMONIUM SULPHATE

The fractions given are the best of a series taken in each case. For details of ammonium sulphate and acetone fractionations see text. Recoveries based on streptomycin-treated extract.

	Fraction	
	$(NH_4)_2SO_4$ 27–45%	*Acetone* 25–50%
Specific activity	11	14.5
% recovery of units of activity	50	90
	Further treatment of fraction above with	
	Acetone 70–80%	$(NH_4)_2SO_4$ 27–45%
Specific activity	17	70
% recovery of units of activity	5	56

By taking only the protein precipitated by higher concentrations of acetone it was possible to obtain enzymes of higher purity but only at the expense of overall yield. In Table II the purification procedure which gave the highest specific activity of InGP synthetase is described. This purification represents a 17-fold increase in specific activity over the starting material, the crude *E. coli* T-8 extract, and 1000-fold over that in extracts of the wild type *E. coli* K-12.

The details of the experiment summarized in Table II are as follows. All operations were carried out at 0–5°. To remove nucleic acids, one-half volume of 20% streptomycin sulphate was added with stirring to *E. coli* T-8 crude extract. Stirring was continued for 10 min after which the mixture was centrifuged at 4,600 × *g* for 10 min. The supernatant was decanted and stored at −15°. 10 ml of treated

TABLE II

PURIFICATION OF INDOLEGLYCEROL PHOSPHATE SYNTHETASE FROM *E. coli* T-8

For details of experiment see text.

	Specific activity	*Purification*	*% units recovered*
Crude extract	10	—	100
Streptomycin-treated and dialyzed*			104
Acetone fractionation			
(a) 0–40 %	5	0.5	39
(b) 40–50 %	17	1.7	26
Ammonium sulphate fractionation of (b)			
30–45 %	84	8.4	5
45–55 %	170	17	6.7

* The protein content after streptomycin treatment could not be determined accurately because residual streptomycin interfered with the protein assay.

extract was dialysed against 1 l of 0.03 *M* Tris buffer pH 7.8 for 2 h changing the buffer after 1 h. The final volume was 11.7 ml and to 10 ml stirred in an ice bath was added 4 ml of acetone, chilled to −15°, over a period of about 0.5–1 min. After further stirring for 1 min the precipitate was collected by centrifugation at 12,000 × *g* for 2 min. The precipitate was taken up in 10 ml of 0.1 *M* Tris buffer pH 7.8 (0–40 % acetone fraction). To the supernatant was added a further 1-ml portion of cold acetone as above and the precipitated protein centrifuged and taken up in 5 ml Tris buffer (40–50 % acetone fraction).

To 4 ml of the 40–50 % acetone fraction 0.87 g $(NH_4)_2SO_4$ (30 % saturation) was added, the resulting solution stirred for 10 min and then centrifuged at 4,600 × *g* for 10 min. The precipitate was taken up in 4 ml 0.1 *M* Tris pH 7–8 and $(NH_4)_2SO_4$ added to the supernatant to give 45 % saturation. After collecting the 30–45 % fraction a further fraction at 45–55 % saturation was collected.

Chromatography

Although it has not yet been possible to obtain appreciable purifications of InGP synthetase by chromatography on DEAE cellulose columns, nevertheless this procedure was employed to obtain fractions for the mixing experiments to be described later. A crude extract of *E. coli* 7-4 (25 ml) was prepared for the column by treatment with $MnCl_2$ (1.5 ml *M* solution/20 ml extract) to remove nucleic acids and then precipitating the enzymes with 50 % saturated $(NH_4)_2SO_4$. The precipitated enzymes were dissolved in 0.1 *M* Tris buffer pH 7.8 and dialysed for 2 h against 0.01 *M* phosphate buffer pH 7.8 plus 10^{-4} glutathione and $2 \cdot 10^{-5}$ *M* pyridoxal phosphate. The latter compounds were added to protect the component B of tryptophan synthetase[8] which was being collected at the same time and they were also included in the eluting buffers. A column of DEAE cellulose 2 cm × 90 cm was used and the linear gradient elution system was 1 l of 0.01 *M* phosphate buffer pH 7.0 in the mixing bottle and an equal volume of 0.4 *M* phosphate pH 8.0 in the inlet bottle. Fractions of 7.5 ml were collected every 18 min and the buffer changed at fraction 173 to elute the B component of tryptophan synthetase. Fig 1 shows that active material was eluted as a well-defined peak.

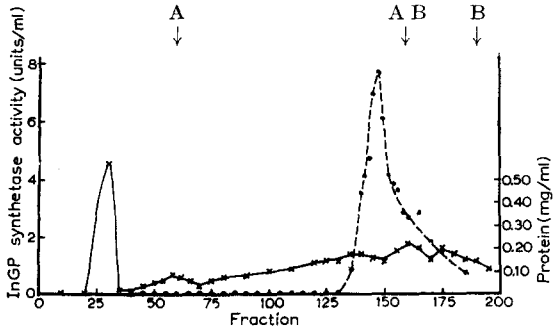


Fig. 1. Chromatography of InGP synthetase on DEAE column. Extract from *E. coli* 7-4. Positions of A and B proteins of tryptophan synthetase as indicated. For details of experiment see text. ●—●, InGP synthetase activity (units/ml); ×—×, protein (mg/ml).

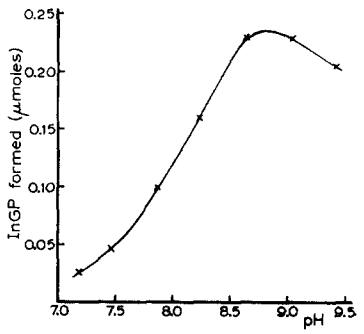


Fig. 2. Effect of pH on activity of InGP synthetase in Tris buffer. For details of test systems (anthranilic deoxyribulotide, 0–6 μmole; InGP synthetase and buffer) see METHODS.

Inhibitors

It had been shown previously[9] that when cultures of *E. coli* are grown in the presence of subinhibitory concentrations of 3-methyl anthranilic acid, anthranilic deoxyribulotide accumulates in the culture medium. This finding indicated that 3-methyl anthranilic acid probably inhibited indoleglycerol phosphate synthetase and using the enzyme assay system described above it was possible to test this directly.

The results of the tests with this compound and with other derivatives of anthranilic acid are shown in Table III. It can be seen that the anthranilic acid analogues fell into 2 groups when tested at a concentration of 10^{-3} *M*, those showing appreciable inhibition and those with little or no effect.

Other inhibitors which were tested in the system given in Table III and the concentrations used together with percentage inhibition are as follows: *p*-chloromercuribenzoate (10^{-5} *M*; 77 %) Versene ($5 \cdot 10^{-2}$ *M*; 32 %; 10^{-2} *M*, 6 %), hydroxylamine (10^{-3} *M*, 17 %; 10^{-4} *M*, 6 %).

Effect of pH on IGP synthetase activity

In Tris buffer a well-defined optimum was shown at pH 8.8 as illustrated in Fig. 2. In phosphate buffer, tested over the range pH 6.5–8.0, the activity shows a steady increase and while at pH 8.8 the activity is greater in Tris than in phosphate buffer, between pH 7 and 8 the activity is much higher in phosphate buffer than in Tris.

Effect of temperature on activity

The enzyme shows a Q_{10} of about 2 between 20° and 30° and there is further increase in activity to 37°. When heated in 0.1 *M* Tris buffer pH 7.8 at 45° for 10 min 75 % of the activity is lost.

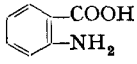
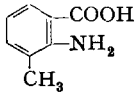
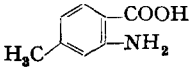
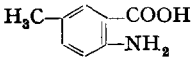
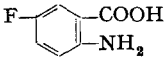
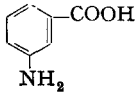
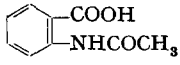
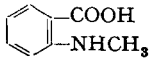
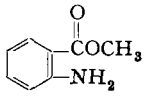
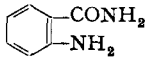
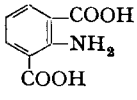
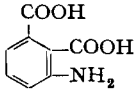
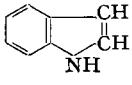
Stability of enzyme

Solutions of IGP synthetase stored at —15° seem to be quite stable. The ammonium sulphate precipitated preparation used for most of the experiments described

TABLE III

INHIBITION OF INDOLEGLYCEROL PHOSPHATE SYNTHETASE BY ANTHRANILIC ACID AND RELATED COMPOUNDS

E. coli T-8 preparation (see METHODS) was incubated in the assay mixture (see text) with and without the inhibitors shown.

Compound	*Structure*	*% inhibition by 10^{-3} M*
Anthranilic acid		31
3-methyl anthranilic acid		40
4-methyl anthranilic acid		36
5-methyl anthranilic acid		25
5-fluoro anthranilic acid		27
3-amino benzoic acid		3
N-acetyl anthranilic acid		2
N-methyl anthranilic acid		5
Phenylglycine *o* carboxylic acid		10 (stimulation)
Methyl anthranilate		0
2-amino benzamide		0
2-amino isophthalic acid		4
3-amino phthalic acid		7 (stimulation)
Indole		5

here showed no decrease in activity over a period of more than 3 months, despite repeated freezing and thawing.

Kinetic studies

Examination of the rate of formation of InGP from anthranilic deoxyribulotide showed that the conversion had the characteristics of a first order reaction. Determination of initial velocities of the reaction were made measuring IGP formation at 2, 4 and 6 min at concentrations of anthranilic deoxyribulotide of $8 \cdot 10^{-5}$, $4.4 \cdot 10^{-5}$ and $2.2 \cdot 10^{-5}$ M. By plotting substrate concentration divided by velocity against velocity a Michaelis constant of $1.75 \cdot 10^{-5}$ M was obtained.

Mixing of fractions obtained during purification procedures

Attempts were made to determine whether the reaction under investigation was carried out by a single enzyme or 2 or more enzymes. One approach employed was to mix various fractions obtained during purification and to examine for stimulation. If 2 enzymes were involved in InGP formation and partial separation achieved at any step in purification, then mixing of 2 such fractions would result in more InGP formation than the sum of the InGP formed by each preparation alone. On no occasion was such a stimulation observed. A typical result obtained on mixing fractions obtained following an acetone fractionation is shown in Table IV. Mixtures were

TABLE IV

InGP SYNTHETASE ACTIVITY OF FRACTIONS OBTAINED DURING ACETONE FRACTIONATION

Fractionation of streptomycin-treated crude extract (see text). Precipitates all dissolved in the same volume of 0.1 M Tris buffer pH 7.8 as the volume of extract fractionated (10 ml) and tested in standard system (see METHODS).

		(μmoles) InGP formed
(a) Crude extract, streptomycin-treated	0.01 ml	1.18
	0–25	0.01
	25–40	0.60
Acetone precipitated fractions (%)	40–50	0.45
(b) (0.01 ml tested)	50–60	0.01
	60–90	0
Total activity		1.07
(c) Mixture of 0.01 ml of each fraction		1.12

made from acetone fractionations (before and after ammonium sulphate fractionation) and following both ammonium sulphate fractionation and chromatography on DEAE cellulose. In the latter case fractions from the front and trailing edges of the InGP synthetase peak were mixed. In one experiment when the total recovery of enzymic activity was very low, all the fractions were pooled and treated with ammonium sulphate (50% saturation) but this did not result in a recovery of more activity than was found in all the individual fractions.

The addition of heated crude extract or yeast extract to material collected from the column failed to increase its activity.

Mixing of crude extracts obtained from a number of strains of E. coli

Another method employed in examining whether InGP synthetase was a single enzyme was to mix the crude extracts of a number of different mutants of *E. coli*, each alone unable to carry out the conversion of anthranilic deoxyribulotide to InGP. If this reaction requires 2 enzymes then some mutants may lack one and other mutants lack the second. Mixing of extracts of members of these 2 groups would result in activity for the overall reaction.

Fourteen different mutants of *E. coli* K-12 were chosen, each judged by nutritional and accumulation tests to be blocked between anthranilic deoxyribulotide and InGP. All of these when tested individually were found to be devoid of InGP synthetase activity. These mutants were grown under the conditions described for the preparation of extracts of *E. coli* T-8 and therefore were likely to contain a high concentration of tryptopahn synthetase. The presence of any serine in the crude extracts would result in the conversion of any InGP formed to tryptophan. The formation of tryptophan was prevented by the inclusion in the tests of 10^{-4} *M* hydroxylamine which inhibits this reaction of tryptophan synthetase. An experiment showing the effect of hydroxylamine is given in Table V. A known amount of InGP

TABLE V

THE EFFECT OF HYDROXYLAMINE ON THE REMOVAL OF InGP BY CRUDE EXTRACTS

Basal substrates – Tris buffer pH 8.8, anthranilic deoxyribulotide (for details see METHODS) with additions as shown.

Tube	*1*	*2*	*3*	*4*
E. coli 941 crude extract 0.1 ml	—	+	+	+
InGP synthetase*	+	—	+	+
Hydroxylamine (Final concn. 10^{-4} *M*)	—	+	—	+
InGP formed (μmoles)	0.10	$<$ 0.005	0.06	0.08

* See METHODS ($\equiv$ to about 0.004 ml of crude *E. coli* T-8 extract).

TABLE VI

MIXING OF CRUDE EXTRACTS FROM VARIOUS AUXOTROPHIC STRAINS OF *E. coli* EACH WITH NO InGP SYNTHETASE ACTIVITY

Basal substrates – Tris buffer pH 8.8, anthranilic deoxyribulotide (see METHODS) plus 10^{-4} *M* hydroxylamine with additions as shown.

Mixture of extracts of 7 strains of E. coli (0.02 ml each)	*Indole formed (μmoles)*	
	Mixture alone	*Mixture + InGP synthetase**
A	$<$ 0.05	0.10
B	$<$ 0.05	0.10
C	$<$ 0.05	0.10
D	$<$ 0.05	0.10
E. coli T-8 extract (0.004 ml)		0.11

* T-8 extract ((0.004 ml).

synthetase was included in the test. It can be seen that the inclusion of the hydroxylamine did prevent the removal of InGP. The small amount that was removed could be accounted for by the formation of indole from InGP, a reaction which is much slower than the InGP to tryptophan reaction[10] and would be carried out in the presence of hydroxylamine.

The 14 mutant extracts were mixed in groups of 7 at a time so that each extract was tested against every other one. These tests would have detected an activity of 1% of that in *E. coli* T-8 assuming the same specific activity. In no mixture of extracts was there any detectable activity (Table VI). In all experiments a control tube for each mixture was included which contained a known amount of InGP synthetase to detect inhibitory substances in the extracts. No inhibition was detected and the results of one such experiment are summarized in Table VI.

Specific activities of InGP synthetase and related enzymes in crude extracts

It has recently been reported[11] that 4 enzymes concerned in histidine biosynthesis showed co-ordinate repression (release). That is, when mutant or wild-type cells were grown under various conditions the specific activities of the enzymes studied were all increased in the same extract, to a similar extent. The levels of the enzymes of the tryptophan synthetase system, as well as InGP synthetase, were examined in several strains to determine whether co-ordinate repression was also evident in the tryptophan pathway. The specific activities of the enzymes from the various mutants studied are shown in Table VII. It can be seen that the different proteins are not increased equally in the mutants derived from *E. coli* K-12. Thus with *E. coli* A-34 (compared with wild type) IGP synthetase is increased 34-fold, protein A 20-fold and protein B 14-fold. On the other hand, InGP synthetase and protein A in *A. coli* 7–4 seem to be increased to the same extent. From such limited data it appears that co-ordinate repression release may not be an invariable rule.

TABLE VII

SPECIFIC ACTIVITIES OF TRYPTOPHAN SYNTHETASE AND InGP SYNTHETASE OF CRUDE EXTRACTS OF WILD TYPE AND MUTANT STRAINS OF *E. coli*

Specific activity = number of units (0.1 μmole of substrate removed or end product produced in 20 min) per mg protein. Tryptophan synthetase (TS) activity measured in the indole → tryptophan reaction.

Strain of E. coli	*Specific activity of*		
	InGP synthetase	*Protein A of T.S.**	*Protein B of T.S.**
K_{12} wild type parent	0.167	1.56	1.50
K_{12} (indole)**	0.172	1.41	1.33
T-8	10	***	36
A 34§	5.7	30.8	21.6
518 wild type parent	0.2	1.4	0.85
7-4§	11.7	83	***

* For assay methods see ref. 10, 11.
** Cells grown on medium containing 2 μg indole/ml.
*** Activity not measurable in indole → tryptophan reaction because of mutation.
§ Cells grown on medium containing 5 μg L-tryptophan/ml.

DISCUSSION

InGP synthetase has proven to be an enzyme which is fairly stable and can readily be assayed in cell-free extracts of *E. coli*. *Escherichia coli* T-8 is a mutant particularly suited to the study of this enzyme since it lacks the A protein of tryptophan synthetase and is therefore unable to convert the product of the reaction, InGP, to either indole or tryptophan. However, with extracts of other types of mutants the interference of the conversion of InGP to tryptophan can be prevented by the inclusion of hydroxylamine which inhibits this reaction, and the small amount of indole formed does not appreciably affect the results.

To obtain enzyme preparations with a high specific activity a most important step is the control of the growth of the tryptophan auxotrophs by limiting the available tryptophan. This method has been employed in the study of other enzymes concerned in tryptophan biosynthesis[2]. In the present case repression release resulted in cell extracts in which there was 60 times as much enzyme per unit protein as that obtained from wild-type cells. Further purification by acetone and ammonium sulphate fractionation gave an additional 17-fold increase in specific activity but only at the expense of a large loss of enzyme activity.

The inhibitory action of anthranilic acid and its analogues on InGP synthetase is of considerable interest as it might have been expected that the analogues would inhibit the metabolism of anthranilic acid but not that of the next intermediate, the deoxyribulotide. This inhibitory effect accounts for the observations of LESTER (see above) on the accumulation of anthranilic deoxyribuloside in the presence of 3-methylanthranilic acid. The other ring-substituted anthranilic acids would not be expected to cause any such accumulation since they are metabolized and probably converted to the corresponding substituted tryptophans. Under the conditions of the cell-free experiments described here, metabolism is not possible and inhibition can be examined. It appears from the inhibition tests with the anthranilic acid analogues that one requirement for inhibition is that both the carboxyl group and the amino group be unsubstituted.

The inhibitory action of anthranilic acid itself on InGP synthetase may explain the ability of this compound to inhibit bacterial growth. RYDON[12] observed that some strains of *Salmonella typhi* were inhibited to some extent by $8 \cdot 10^{-5}\,M$ anthranilic acid. Also it is known that $5 \cdot 10^{-4}\,M$ anthranilic acid will inhibit the growth of a tryptophan auxotroph which will grow well in $10^{-4}\,M$ anthranilic acid (GIBSON, unpublished results).

In the experiments described no requirements for coenzymes or metals have been demonstrated. The addition of yeast extract or heated cell extracts were tried on several occasions but were not found to stimulate InGP formation.

It appears from the findings reported here that the conversion of anthranilic deoxyribulotide to InGP is probably carried out by a single enzyme. The mixing of various fractions obtained during purification did not give any stimulation of activity, indicating that if 2 or more enzymes are involved they fractionate identically. The mixing experiments with extracts obtained from 14 auxotrophic strains of *E. coli* unable to convert anthranilic deoxyribulotide to InGP strongly supports the view that only one enzyme is concerned in the reaction. In a similar application of auxotrophs in the conversion of anthranilic acid to InGP, SMITH AND YANOFSKY[1] were

readily able to resolve the overall reaction into a sequence of at least 2 reactions by using mixtures of mutant extracts.

The mechanism of removal of water and carbon dioxide and formation of a pyrrole ring by one enzyme will be an interesting subject for future investigation.

ACKNOWLEDGEMENTS

This research was supported by grants from the U.S. Public Health Service and the National Science Foundation. One of us (F.G.) is indebted to the Carnegie Foundation of New York for a travel grant which made this work possible and to the Department of Biological Sciences at Stanford University for its hospitality during leave from the University of Melbourne. We wish to thank Dr. M. GIBSON for her assistance with some of the experiments.

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