Katchalski, E., Fasman, G. D., Simons, E., Blout, E. R., Gurd, F. R. N. & Koltun, W. L. (1960). *Arch. Biophys. Biochem.* **88**, 361.
Koltun, W. H. & Gurd, F. R. N. (1960). *J. Amer. chem. Soc.* **81**, 301.
Koltun, W. H., Ng, L. & Gurd, F. R. N. (1963). *J. biol. Chem.* (in the Press).
Krebs, E. G. (1955). In *Methods in Enzymology*, vol. 1, p. 407. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Kuby, S. A., Noda, L. & Lardy, H. A. (1954*a*). *J. biol. Chem.* **209**, 191.
Kuby, S. A., Noda, L. & Lardy, H. A. (1954*b*). *J. biol. Chem.* **210**, 65.
Mahowald, T. A. & Kuby, S. A. (1960). *Fed. Proc.* **19**, 46.
Mahowald, T. A., Noltmann, E. A. & Kuby, S. A. (1962). *J. biol. Chem.* **237**, 1535.
Nihei, T., Noda, L. & Morales, M. F. (1961). *J. biol. Chem.* **236**, 3203.
Noda, L., Kuby, S. A. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 203.
Noltmann, E. A., Mahowald, T. A. & Kuby, S. A. (1962). *J. biol. Chem.* **237**, 1146.
Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.
Rabin, B. R. & Watts, D. C. (1960). *Nature, Lond.*, **188**, 1163.
Samuels, A. J., Nihei, T. & Noda, L. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1992.
Smith, R. M. & Alberty, R. A. (1956). *J. Amer. chem. Soc.* **78**, 2376.
Smithies, O. (1959). *Advanc. Protein Chem.* **14**, 65.
Watts, D. C. (1961). Ph.D. Thesis: University of London.
Watts, D. C. & Rabin, B. R. (1962). *Biochem. J.* **85**, 507.
Watts, D. C., Rabin, B. R. & Crook, E. M. (1961). *Biochim. biophys. Acta*, **48**, 380.
Watts, D. C., Rabin, B. R. & Crook, E. M. (1962). *Biochem. J.* **82**, 412.

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# The Conversion of Shikimic Acid into Certain Aromatic Compounds by Cell-Free Extracts of *Aerobacter aerogenes* and *Escherichia coli*

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One of the major unsolved problems in the biosynthesis of aromatic compounds by microorganisms is the process by which shikimic acid 5-phosphate is converted into anthranilic acid. This involves the formation of an aromatic ring and the introduction of nitrogen into the molecule and might therefore be expected to take place in several steps. Although tryptophan auxotrophs that appear to have a metabolic lesion in this region of the pathway have been known for many years (Yanofsky, 1955; Tatum, Bonner & Beadle, 1943; Nyc, Mitchell, Leifer & Langham, 1949; Gots, Koh & Hunt, 1954), there have been no reports of the accumulation of possible intermediates by such cells. Investigations of accumulations by whole cells of certain tryptophan auxotrophs yielded mixtures of phenolic compounds (Pittard, Gibson & Doy, 1961, 1962; Doy & Gibson, 1961). The relation of these compounds to tryptophan synthesis, however, was not clear, and it was decided to seek further information on the conversion of shikimic acid 5-phosphate into anthranilic acid by using cell-free extracts of bacterial auxotrophs.

Srinivasan (1959) reported briefly on the enzymic synthesis of anthranilic acid from shikimic acid 5-phosphate by cell-free extracts of a mutant strain of *Escherichia coli*. He found that, with shikimic acid 5-phosphate as substrate, glutamine and NAD, $NADH_2$ or NADP were required for optimum synthesis.

The present work confirms and extends the results of Srinivasan. The experimental system used was also suitable for examining the biosynthesis of phenylalanine and tyrosine. Part of this work has been briefly reported (Morgan, Gibson & Gibson, 1962).

## MATERIALS AND METHODS

*Organisms.* Various auxotrophic strains of *Escherichia coli* and *Aerobacter aerogenes* were used. These strains were obtained by penicillin selection (Davis, 1948; Lederberg & Zinder, 1948) after ultraviolet irradiation. The wild-type strain of *A. aerogenes* was first irradiated and a non-capsulated variant isolated, this variant being used as the parent strain for the auxotrophs used. Some details of the strains used are set out in Table 1.

All strains were maintained on meat infusion–agar slopes and subcultured monthly.

*Preparation and fractionation of cell extracts.* The cells were grown in the citric acid–mineral salts mixture described by Vogel & Bonner (1956), supplemented with 0·16% of glucose, 0·005% of acid-hydrolysed casein and indole (2 μg./ml.). The concentration of indole was made limiting to encourage de-repression on the tryptophan pathway.

Table 1. *Strains of auxotrophs*

Substances listed under 'Growth requirement' are the supplements needed for growth in glucose–mineral salts medium.

| Auxotroph | Growth requirement | Compound(s) accumulated by cell suspensions | Probable site of metabolic lesion |
|---|---|---|---|
| *A. aerogenes* T17 | Tryptophan or indole | Anthranilic acid | *N*-(5-Phosphoribosyl)anthranilic acid ⟶ 1-(*o*-carboxyphenylamino)-deoxyribulose 5-phosphate* |
| *A. aerogenes* NC3 | Tryptophan or indole or anthranilic acid | Phenols† | Between the last intermediate common to the synthesis of all the aromatic amino acids and anthranilic acid |
| *E. coli* W2–5‡ | Tryptophan or indole | Anthranilic acid | Anthranilic acid ⟶ *N*-(5-phospho-ribosyl)anthranilic acid* |

* See Doy, Rivera & Srinivasan (1961). † See Pittard, Gibson & Doy (1961). ‡ See Discussion.

Quantities (1 l.) of medium were inoculated with about $10^9$ cells from a 6–8 hr. shaken meat-infusion broth culture. The 1 l. cultures in 2 l. flasks were then grown at 37° while aerated (400 ml./min.) through a central sintered-glass filter; alternatively, 1 l. quantities were shaken on a rotary shaker at 30°. Flasks were incubated overnight. No differences were noted in enzyme preparations from the cells grown at different temperatures.

The cells from each flask were spun down and washed once in about 250 ml. of cold 0·9% NaCl solution. In early experiments cells were smashed in a Hughes (1951) press at approximately −20° and taken up in 10 ml. of 0·1 M-tris buffer, pH 7·8. The resulting highly viscous material was centrifuged at 24500*g* for 30 min., the clear supernatant decanted (crude extract) and stored at −20°. In later experiments the cells were smashed in a MSE–Mullard 500 w ultrasonic disintegrator after resuspending the washed cells in 4 ml. of 0·1 M-tris buffer, pH 7·8/g. wet wt. of cells. After centrifuging as above the supernatants (crude extract) were stored at −20°. All the experiments described involving fractionation of enzymes were carried out after ultrasonic treatment.

Nucleic acids were removed from the crude extract by the addition of 1·5 ml. of M-$MnCl_2$/20 ml. of extract, stirring for 10 min., centrifuging (24000*g* for 10 min.) and decanting the supernatant.

Ammonium sulphate fractionations were carried out by adding solid $(NH_4)_2SO_4$ to the required saturation (72 g./100 ml. was regarded as 100% saturation), stirring for 10 min., centrifuging (24000*g* for 10 min.), decanting the supernatant and taking up the deposit in 0·1 M-tris buffer, pH 7·8, and dialysing against 0·01 M-tris buffer, pH 7·8, for 2 hr. All the operations described above were carried out at 0–5°.

*Reagents.* The reagents used with the exception of shikimic acid 5-phosphate, which was kindly given by Dr B. Davis, were obtained commercially and not further purified. Ribose 5-phosphate solutions were made by treating the barium salt with a slight excess of $Na_2SO_4$, and removing and washing the precipitate by centrifuging. Boiled crude extract was made by heating crude extract prepared as above for 5–10 min. at 100°. Buffer recipes were taken from Dawson, Elliott, Elliott & Jones (1959).

*Enzyme reactions.* The mixture of enzyme and substrate was usually in a final volume of 1 ml. All tests were carried out at 37°.

### *Analytical methods*

*Anthranilic acid.* In the earlier experiments anthranilic acid was estimated by the Bratton & Marshall (1939) test for diazotizable amines as described by Gibson & McDougall (1961). This method was not specific and it was found more convenient to extract anthranilic acid into ethyl acetate and estimate it spectrophotometrically. To 1 ml. of test solution was added 0·1 ml. of N-HCl, followed by 4 ml. of ethyl acetate. The tubes were mixed by inversion 10–15 times and centrifuged at a low speed to separate the solvent. The extinction of the ethyl acetate layer was measured against an ethyl acetate blank at 336 mμ. The concentration of anthranilic acid was calculated by assuming an extinction coefficient at 336 mμ of 4900 (Doy & Gibson, 1959). Except for the experiment of Fig. 3, the extraction method was used in the work described.

*Phenylpyruvic acids.* The phenylpyruvic acids were detected after acidification and extraction of reaction mixtures with ethyl acetate as in the anthranilic acid estimations. Immediate spectra and spectra after overnight incubation of the ethyl acetate extracts at 30° were recorded. A spectrum with a peak at 288 mμ was obtained after incubation. Extinction at 288 mμ for a given concentration of phenylpyruvic acid varied somewhat from experiment to experiment although it was consistent in any one experiment. Therefore the amount of phenylpyruvic acids present in ethyl acetate extracts is expressed in terms of the extinction at 288 mμ rather than as concentrations.

*Adenosine 5′-triphosphate.* Adenosine triphosphate was estimated by a micro-method using a luciferin–luciferase extract of 'Live-desiccated' Firefly Tails (Sigma Chemical Co.) and following the method given with the extract. A calibration curve was prepared each time the test was performed. The presence of 1 μmole of ATP in this test produced approximately 35% transmission.

*Shikimic acid.* Two methods were used for the estimation of shikimic acid: colorimetric and microbiological. The colorimetric assay was that described by Gaitonde & Gordon (1958). To 3 ml. of solution containing 2–10 μg. of shikimic acid was added 0·5 ml. of periodic acid (1%, w/v) and the mixture allowed to stand 3 hr. A portion (0·5 ml.) of N-NaOH, followed immediately by 0·3 ml. of 0·1 M-glycine, was added with shaking. Extinction at 380 mμ was measured as soon as possible. Despite the glycine, which is reported to stabilize the chromophore, extinction

was not steady, so readings were made as soon as possible and a standard curve was prepared for each assay.

The microbiological assay was essentially that described by Davis & Mingioli (1953), except that the Vogel & Bonner (1956) medium was used. In experiments where it was intended to estimate both shikimic acid and shikimic acid 5-phosphate separately, experimental samples of 0·2 ml. or less were autoclaved in $Na_2CO_3$ solution (Davis & Mingioli, 1953) and then sterile medium, supplemented with L-tyrosine (0·1 mM), DL-phenylalanine (0·2 mM) and glucose (0·16%), was added. The inoculum was 5 $\mu$l. of a 5 hr. culture of *E. coli* 83–1. After incubation at 37° for 18 hr. extinction of the culture at 450 m$\mu$ was recorded.

*Shikimic acid 5-phosphate.* The estimation of shikimic acid 5-phosphate was carried out by treating samples with alkaline phosphatase and estimating the shikimic acid liberated. Experimental samples (0·1 ml.) containing 0·02–0·1 $\mu$mole of shikimic acid 5-phosphate were incubated with 0·1 ml. of alkaline phosphatase solution (1 mg./ml. in 0·1 M-veronal buffer, pH 10·1) for 30 min. at 37°. The volume was made up to 3 ml. with water and the samples were assayed for shikimic acid.

Alternatively a sample containing 0·02–0·1 $\mu$mole of shikimic acid 5-phosphate was autoclaved (15 lb./in.$^2$ for 30 min.) with an equal volume of 0·2 N-HCl. Shikimic acid 5-phosphate is 25–30% hydrolysed under these conditions to yield shikimic acid. A control tube for each sample was autoclaved with an equal volume of 0·2 M-$Na_2CO_3$. The volumes were then made up to 3 ml. with water and the samples assayed for shikimic acid.

With mixtures of shikimic acid and shikimic acid 5-phosphate, assay for the former compound before and after hydrolysis allowed estimation of both compounds.

*Protein.* Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

*Chromatography.* Ascending paper chromatography was carried out on Whatman no. 1 paper as described by Pittard *et al.* (1961). The following single-phase solvent systems were used: methanol–butan-1-ol–benzene–water (2:1:1:1, by vol.); benzene–acetic acid–water (125:72:3, by vol.); butan-1-ol–acetic acid–water (12:3:5, by vol.).

*Spectrophotometry.* A Unicam SP. 500 spectrophotometer was used. All spectra were read at 5 m$\mu$ intervals or less.

## RESULTS

### *Conversion of shikimic acid into anthranilic acid by crude cell extracts*

Preliminary experiments were based on the results of Srinivasan (1959). In contrast with Srinivasan's results, our cell extracts converted shikimic acid as efficiently as they converted shikimic acid 5-phosphate into anthranilic acid. Further, the addition of a sugar phosphate was found to stimulate the conversion.

As is shown in Table 2, the conversion of shikimic acid into anthranilic acid by crude cell-free extracts was found to be stimulated by magnesium chloride, L-glutamine, ATP, NAD and ribose 5-phosphate. The rate of anthranilic acid production and the utilization of shikimic acid and shikimic acid 5-phosphate are shown in Figs. 1 and 2. Anthranilic acid production began after a short lag and generally ceased after 1–1½ hr. The experiment illustrated in Fig. 1 shows an 88% conversion of the shikimic acid utilized into anthranilic acid, but yields were frequently lower than this. It was found consistently that shikimic acid and shikimic acid 5-phosphate were equivalent as substrates. All extracts were capable of rapidly phosphorylating shikimic acid, and, during the formation of anthranilic acid from the complete system, shikimic acid 5-phosphate accumulated and was later removed (Fig. 2). Omission of ribose 5-phosphate or NAD from the complete system decreased by approximately 50% the amount of shikimic acid 5-phos-

Table 2. *Requirements for the conversion of shikimic acid into anthranilic acid by crude cell extracts*

The complete system contained 50 $\mu$moles of tris–HCl buffer, pH 8·2, 1 $\mu$mole of shikimic acid, 5 $\mu$moles of $MgCl_2$, 5 $\mu$moles of L-glutamine, 1 $\mu$mole of ATP, 1 $\mu$mole of NAD, 1 $\mu$mole of ribose 5-phosphate. All tubes contained T17 cell extract (2 mg. of protein) in a final volume of 1 ml. Incubated for 60 min. at 37°.

| Substrate omitted | Anthranilic acid formed ($\mu$m-moles) |
|---|---|
| None (complete system) | 540 |
| Shikimic acid | 26 |
| $MgCl_2$ | 170 |
| L-Glutamine | 26 |
| ATP | 30 |
| NAD | 170 |
| Ribose 5-phosphate | 34 |

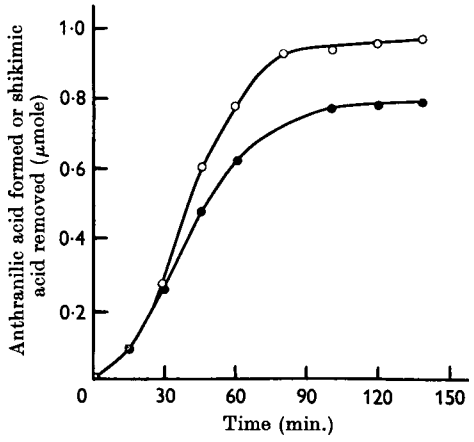


Fig. 1. Removal of shikimic acid and formation of anthranilic acid. Crude extract of strain T17 (2 mg. of protein/ml.) was incubated in the complete reaction mixture of Table 2 at 37°. Samples were removed at intervals and assayed. ●, Anthranilic acid; ○, total shikimic acid (shikimic acid plus shikimic acid 5-phosphate). Shikimic acid was determined by the method of Gaitonde & Gordon (1958).

phate utilized. Ribose 5-phosphate could be replaced by fructose 6-phosphate or glucose 6-phosphate.

*Requirement for ATP.* The effect of ATP on anthranilic acid production from shikimic acid was variable. Table 2 shows an 18-fold decrease in anthranilic acid production when ATP was omitted from the system, and on some occasions ATP had no effect or even an inhibitory effect on anthranilic acid production. Fig. 3 shows the effect of ATP as determined on two separate occasions. In neither case was the requirement for ATP absolute, and increase in ATP concentration above approximately 0·5 μmole/ml. was inhibitory. Analysis by the luciferin–luciferase method showed that one freshly prepared extract contained 0·03 μmole of ATP/ml., and another extract that had been stored for about 3 months at −20° contained < 0·005 μmole/ml. These amounts are far too low to account for the consistent phosphorylation of shikimic acid in the absence of added ATP.

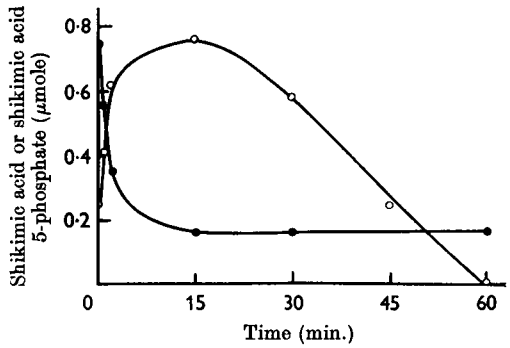


Fig. 2. Removal of shikimic acid and appearance of shikimic acid 5-phosphate. Conditions were given in Table 2. Samples were removed at intervals and assayed for ●, shikimic acid and ○, shikimic acid 5-phosphate. Shikimic acid was determined by the method of Gaitonde & Gordon (1958). Shikimic acid 5-phosphate was determined after treatment with alkaline phosphatase to liberate shikimic acid.

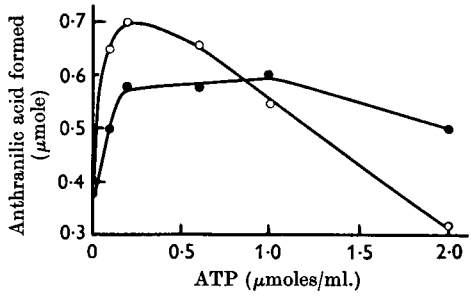


Fig. 3. Requirement of ATP for anthranilic acid formation from shikimic acid. Crude extract of strain T17 (●, 2 mg. of protein/ml./hr.; ○, 1·3 mg. of protein/ml./45 min.) was incubated in the complete reaction mixture minus ATP (see Table 2) at 37° for the times indicated. ATP was added to give the concentrations shown.

*Requirement for L-glutamine.* L-Glutamic acid or ammonium chloride either singly or together did not replace the requirement for L-glutamine in the complete system for anthranilic acid production (Table 3). Progressive stimulation of anthranilic acid production was observed for concentrations of L-glutamine up to 4 mM; 10 mM concentration had no inhibitory effect.

*Requirement for NAD.* The requirement for NAD was not absolute with crude extracts. There was no further stimulation of anthranilic acid production above 0·2 mM-NAD, and 2 mM was not inhibitory. NAD could be replaced by $NADH_2$ in the complete system, and the addition of $NADH_2$ to the complete system stimulated slightly (Table 4).

*Ribose 5-phosphate and related compounds as substrates.* Srinivasan (1959) reported that some extracts were inactive unless they were fortified with yeast extract, suggesting a possible requirement for another cofactor. In the present studies it was found that phosphorylated sugars stimulated

Table 3. *Comparison of L-glutamine, L-glutamic acid and ammonium chloride as substrates for the conversion of shikimic acid into anthranilic acid by crude cell extracts*

All tubes contained T17 cell extract (1·3 mg. of protein) incubated as in Table 2 except that glutamine was replaced by the additions (5 μmoles/ml.) shown.

| Additions | Anthranilic acid formed (μm-moles) |
|---|---|
| L-Glutamine | 170 |
| L-Glutamic acid | 25 |
| $NH_4Cl$ | 39 |
| L-Glutamic acid + $NH_4Cl$ | 64 |

Table 4. *Effect of reduced nicotinamide–adenine dinucleotide and phosphoenolpyruvic acid on the conversion of shikimic acid into anthranilic acid by crude cell extracts*

All tubes contained 50 μmoles of tris–HCl buffer, pH 8·2, 1 μmole of shikimic acid, 1 μmole of ATP, 5 μmoles of L-glutamine, 5 μmoles of $MgCl_2$, T17 cell extract (1·5 mg. of protein), in a final volume of 1 ml. Incubated for 60 min. at 37°.

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Ribose 5-phosphate (5 μmoles) | + | + | + | − | + | − |
| Phosphoenolpyruvic acid (2 μmoles) | − | + | − | + | − | + |
| NAD (1 μmole) | + | + | + | + | − | − |
| $NADH_2$ (1 μmole) | − | − | + | − | + | + |
| Anthranilic acid formed (μm-moles) | 500 | 350 | 630 | 19 | 550 | 65 |

shikimic acid 5-phosphate removal and anthranilic acid production by crude extracts of T 17. Ribose 5-phosphate stimulated anthranilic acid production up to 20-fold. Ribose 5-phosphate, glucose 6-phosphate and fructose 6-phosphate had equivalent activity mole for mole. Ribose was inactive. The optimum concentration of phosphorylated sugar was between 0·3 and 3 $\mu$moles/ml. Excess of phosphorylated sugar was inhibitory.

Since 3-enolpyruvylshikimic acid 5-phosphate is considered to be an intermediate in the conversion of shikimic acid into anthranilic acid (Gibson, Gibson, Doy & Morgan, 1962; Rivera & Srinivasan, 1962), it was expected that phosphoenolpyruvic acid might stimulate anthranilic acid production. In crude extracts phosphoenolpyruvic acid was slightly inhibitory (Table 4) but it stimulated anthranilic acid production by partially purified enzyme systems (see below).

*Effect of* pH. In tris hydrochloride buffer, anthranilic acid production is maximal and constant between pH 8 and 9 (Fig. 4). Tris hydrochloride buffer, pH 8·2, was usually used. Borate buffer also appeared to be suitable but inorganic phosphate inhibited strongly. The inhibition was 50% with about 3 mM-phosphate ($Na_2HPO_4$–$NaH_2PO_4$, pH 8·2) in the presence of tris hydrochloride buffer.

*Effect of temperature.* The optimum temperature for anthranilic acid production was 37° over 1 hr. by cells grown either at 30° or 37°.

*Concentration of enzymes.* Fig. 5 illustrates a typical assay of freshly prepared crude extract in the complete system. Below about 0·6 mg. of protein/ml. no detectable anthranilic acid was formed.

### *Conversion of shikimic acid into anthranilic acid by ammonium sulphate-precipitated enzyme preparations*

When the basic conditions for anthranilic acid formation from shikimic acid by crude cell-free extracts had been established, attempts were made to study in more detail the various reactions involved, as there was no information about the number of intermediates concerned and even the general route of biosynthesis was uncertain at that time.

Experiments were carried out firstly with ammonium sulphate-precipitated enzymes of mutant T 17 in which, after removal of nucleic acids by manganous chloride, the proteins precipitated between 0 and 60% saturation with ammonium sulphate were redissolved in tris hydrochloride buffer and dialysed. These preparations were inactive unless boiled cell extract was added to substrates normally used with crude extracts (see Table 1). However, boiled extract was not required if the following mixture of substrates was used: shikimic acid, ATP, magnesium chloride, $NADH_2$, ribose 5-phosphate, phosphoenolpyruvic acid and L-glutamine in tris buffer, pH 8·2 (Table 5). It was not possible to replace $NADH_2$ with NAD.

Fractionation of cell extracts showed that the overall activity was best demonstrated with the proteins precipitated between 30 and 40% saturation with ammonium sulphate.

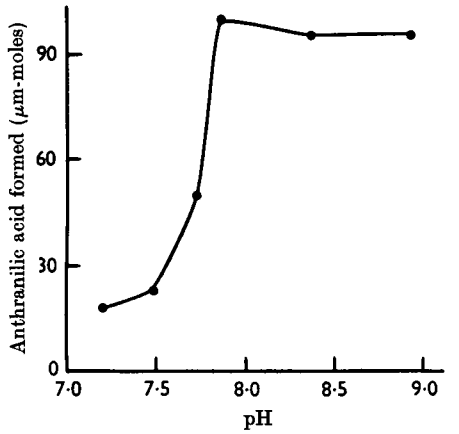


Fig. 4. Effect of pH on anthranilic acid formation. Crude extract of strain T 17 (1·5 mg. of protein/ml.) was incubated at 37° for 30 min. in the complete reaction mixture (see Table 2) minus buffer. Tris buffer (50 $\mu$moles/ml.) was included to obtain the final pH.

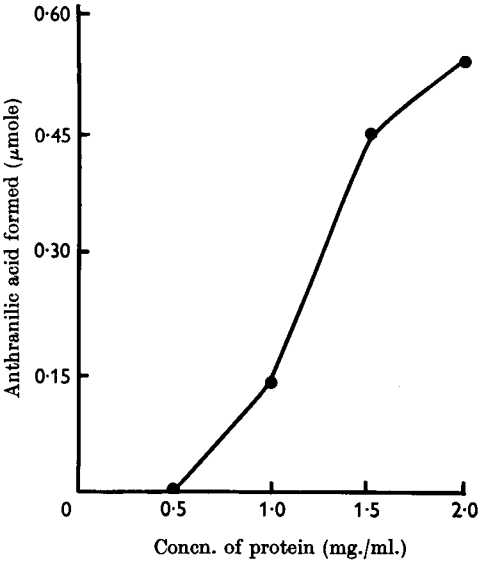


Fig. 5. Titration of crude extract. Anthranilic acid formation was measured after incubation of different concentrations of crude extract of strain T 17 in the complete reaction mixture (see Table 2) for 70 min. at 37°.

*Conversion of shikimic acid into phenylpyruvic acids by crude cell extracts*

Examination of spectra of ethyl acetate extracts of acidified experimental samples indicated that compounds other than anthranilic acid were formed from shikimic acid. If glutamine was omitted from the complete substrates incubated with T17 extracts, or if NC3 extracts were incubated in the presence or absence of glutamine, an unusual type of spectrum was observed in the ethyl acetate. When first examined, the spectrum in the ultraviolet region had no special characteristics, but on storage of the ethyl acetate solutions a pronounced peak developed at 288 m$\mu$ region (Fig. 6) and a shoulder or small peak at 302 m$\mu$. This spectrum has been shown to be due mainly to a mixture of phenylpyruvic acid and 4-hydroxyphenylpyruvic acid (Morgan *et al.* 1962). The rising spectrum was presumably due to the slow conversion of the keto acids into their enol forms in the organic solvent. The spectrum observed for the experimental sample (Fig. 7) closely resembled that obtained from phenylpyruvic acid after a similar extraction procedure. Paper chromatography of such ethyl acetate extracts showed the presence of two components that had the same chromatographic mobilities and colour reactions as phenylpyruvic acid and 4-hydroxyphenylpyruvic acid respectively.

Table 5. *Requirements for anthranilic acid synthesis by an ammonium sulphate-precipitated enzyme preparation from* Aerobacter aerogenes *T* 17

All tubes contained 50 $\mu$moles of tris buffer, pH 8·3, 1 $\mu$mole of shikimic acid 5-phosphate, 5 $\mu$moles of $MgCl_2$, 2·5 $\mu$moles of ribose 5-phosphate, 5 $\mu$moles of L-glutamine, 0–60% ammonium sulphate-precipitated enzyme preparation (strain T17, 1 mg. of protein), in a final volume of 1 ml. Incubated for 75 min. at 37°.

| Addition | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Phosphoenolpyruvic acid (2 $\mu$moles) | + | + | − | + |
| $NADH_2$ (1 $\mu$mole) | + | + | + | − |
| NAD (1 $\mu$mole) | + | − | − | − |
| Anthranilic acid formed ($\mu$m-moles) | 475 | 430 | 133 | 5 |

The colour reactions and mobilities of phenylpyruvic acid and 4-hydroxyphenylpyruvic acid are summarized in Table 6.

Concentrates of ethyl acetate extracts of experimental samples also contained several unidentified compounds that were detected after chromatography by reaction with dinitrophenylhydrazine, silver nitrate and *p*-nitroaniline reagents. 4-Hydroxybenzoic acid produces a cherry-red colour with *p*-nitroaniline reagent. Under the usual reaction conditions T17 and NC3 cell extracts produce a compound that, after acidification and extraction into ethyl acetate, gave $R_F$ values and *p*-nitroaniline

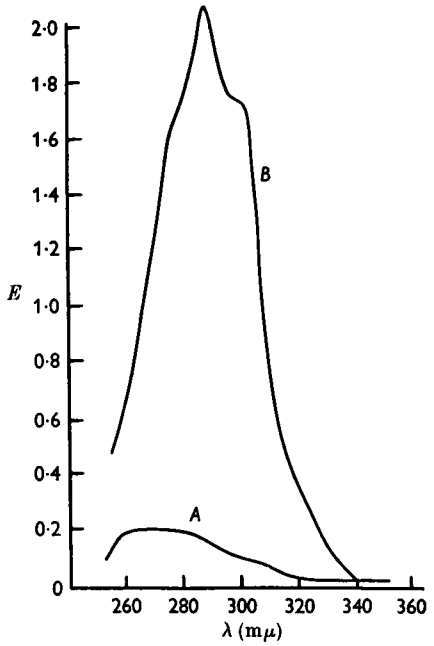


Fig. 6. Appearance of rising spectrum in ethyl acetate extract. Crude extract of strain NC3 (1·7 mg. of protein/ml.) was incubated for 75 min. at 37° in the complete reaction mixture (see Table 2) minus glutamine. After acidification and extraction with 4 ml. of ethyl acetate, the spectrum was recorded: *A*, as soon as possible; *B*, after 16 hr. at room temperature.

Table 6. *Chromatographic properties of phenylpyruvic acid and 4-hydroxyphenylpyruvic acid*

| | $R_F$ | | Colour reaction | | |
|---|---|---|---|---|---|
| | In benzene–acetic acid–water | In methanol–butan-1-ol–benzene–water | With *p*-nitroaniline | With 2,4-dinitrophenylhydrazine | With $AgNO_3$ |
| Phenylpyruvic acid | 0·94 | 0·78 | Yellow–green to grey–green (1 hr.) | Yellow | Brown |
| 4-Hydroxyphenylpyruvic acid | 0·43 | 0·67 | Yellow–pink (diffuse spot) | Yellow | Brown |

reactions indistinguishable from those of 4-hydroxybenzoic acid in the three solvents used.

*Requirements for the production of the phenylpyruvic acids by crude extracts.* Shikimic acid, ribose 5-phosphate (or other sugar phosphates) and NAD were required for, and magnesium chloride stimulated, the production of the phenylpyruvic acids (Table 7). The requirement for ATP was variable, as for anthranilic acid production from shikimic acid. With extracts from strain T17 or NC3, the addition of L-glutamine decreased the amount of the phenylpyruvic acids detected. Fig. 7 illustrates that extracts of strain T17 converted shikimic acid into anthranilic acid in the presence of L-glutamine, and into the phenylpyruvic acids in the absence of glutamine.

*Production of the phenylpyruvic acids in the presence of L-tryptophan.* Several amino acids have been shown to inhibit an early reaction specific to their own formation. Moyed (1960) showed that L-tryptophan inhibited the conversion of shikimic acid 5-phosphate into anthranilic acid by extracts of *E. coli* W in a system similar to that used by Srinivasan (1959). Experiments with *A. aerogenes* T17 extracts have shown that this inhibition was accompanied by the formation of the phenylpyruvic acids. The effect of addition of L-tryptophan (1 $\mu$mole/ml.) to the complete system for anthranilic acid production was similar to the effect of omission of glutamine, as shown in Fig. 6 (see also Morgan *et al.* 1962). The effect of L-tryptophan on anthranilic acid production and production of the phenylpyruvic acids is shown in Fig. 8. The reactions were performed in the presence and absence of hydroxylamine. Hydroxylamine was added in an attempt to prevent the phenylpyruvic acids being converted into phenylalanine and tyrosine, and also to prevent tryptophan breakdown. In the presence and absence of tryptophan, addition of hydroxylamine considerably increased the amount of phenylpyruvic acids detected.

*Rate of production of the phenylpyruvic acids.* Studies on the conversion of shikimic acid into the phenylpyruvic acids were complicated by the fact that these compounds were rapidly removed by cell extracts. The higher the enzyme activity the more rapidly the phenylpyruvic acids appeared and disappeared (Fig. 9). Extracts of strain T17 grown in the presence of excess of L-phenylalanine and L-tyrosine produced and removed the phenylpyruvic acids at rates similar to those of cells grown in the absence of these two amino acids.

### *Conversion of shikimic acid into phenylpyruvic acids by ammonium sulphate-precipitated enzyme preparations*

As for the production of anthranilic acid, a 0–60% ammonium sulphate-precipitated enzyme

Table 7. *Requirements for the conversion of shikimic acid into phenylpyruvic acid and 4-hydroxyphenylpyruvic acid by crude cell extract*

The complete system contained 50 $\mu$moles of tris–HCl buffer, pH 8·3, 1 $\mu$mole of shikimic acid, 5 $\mu$moles of $MgCl_2$, 1 $\mu$mole of ATP, 1 $\mu$mole of NAD, 3 $\mu$moles of ribose 5-phosphate, NC3 cell extract (1·7 mg. of protein), in a final volume of 1 ml. Incubated for 75 min. at 37°. The phenylpyruvic acids were detected after acidifying 0·5 ml. of reaction mixture with 0·4 N-HCl (0·1 ml.), and extracting with 4 ml. of ethyl acetate. The spectrum of the ethyl acetate layer was recorded after 16 hr. at room temperature.

| Substrate omitted | Phenylpyruvic acids formed ($E_{288\,m\mu}$) |
|---|---|
| None (complete system) | 0·97 |
| Shikimic acid | 0·0 |
| $MgCl_2$ | 0·75 |
| ATP | 0·13 |
| NAD | 0·03 |
| Ribose 5-phosphate | 0·0 |
| None (L-glutamine added, 1 $\mu$mole/ml.) | 0·22 |

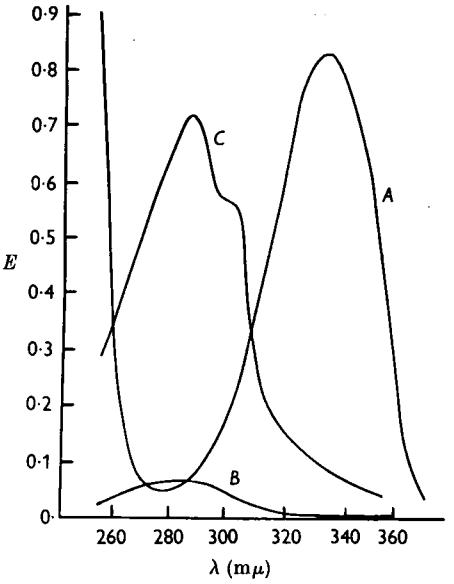


Fig. 7. Formation of phenylpyruvic acids when glutamine was omitted from reaction mixture for anthranilic acid formation. Tube 1 contained the complete reaction mixture (see Fig. 1). In tube 2 glutamine was omitted. Both tubes contained crude T17 extract (1·7 mg. of protein/ml.) and were incubated for 45 min. at 37°. After acidification samples (1 ml.) were extracted with 4 ml. of ethyl acetate and spectra of the ethyl acetate layers were recorded immediately and after 16 hr. at 30°. *A*, Tube 1 (with glutamine), spectra before and after storage; *B*, tube 2 (without glutamine), immediate spectrum; *C*, tube 2, spectrum after storage.

preparation did not convert shikimic acid into the phenylpyruvic acids in the substrate mixture adequate for crude extracts. Activity could be restored to such an enzyme preparation by the addition of boiled crude extract. In the presence of boiled extract it was found that phosphoenolpyruvic acid plus $NADH_2$ could replace ribose 5-phosphate and NAD. Some ammonium sulphate-precipitated enzyme preparations formed phenylpyruvic acids in the absence of boiled extract when phosphoenolpyruvic acid, $NADH_2$ and ribose 5-phosphate were present (Table 8). Other preparations did not produce phenylpyruvic acids in the absence of boiled extract. Most of the preparations that were active in the absence of boiled extract lost this activity on aging.

Phosphoenolpyruvic acid and $NADH_2$ were both required for production of the phenylpyruvic acids in the absence of boiled extract (Table 9). The requirements for sugar phosphate appeared to increase as the precipitated enzyme preparations aged. NAD did not replace $NADH_2$, and the addition of NAD to a reaction mixture containing $NADH_2$ did not stimulate production of phenylpyruvic acids.

Both shikimic acid and shikimic acid 5-phosphate were suitable substrates for production of the phenylpyruvic acids. The extent of the requirement for ATP when shikimic acid was used as substrate was variable. Ribose 5-phosphate could be replaced by glucose 6-phosphate or fructose 6-phosphate (Table 10).

Ammonium sulphate (0–60 %)-precipitated enzyme preparations of NC3 extracts were capable of producing and removing the phenylpyruvic acids both in the presence and absence of L-glutamine.

*Effect of* pH. The conversion of shikimic acid into the phenylpyruvic acids occurred over the range pH 7·0–8·8. The effect of pH was tested with 0–60 % ammonium sulphate-precipitated enzyme preparation in the presence of the complete substrates

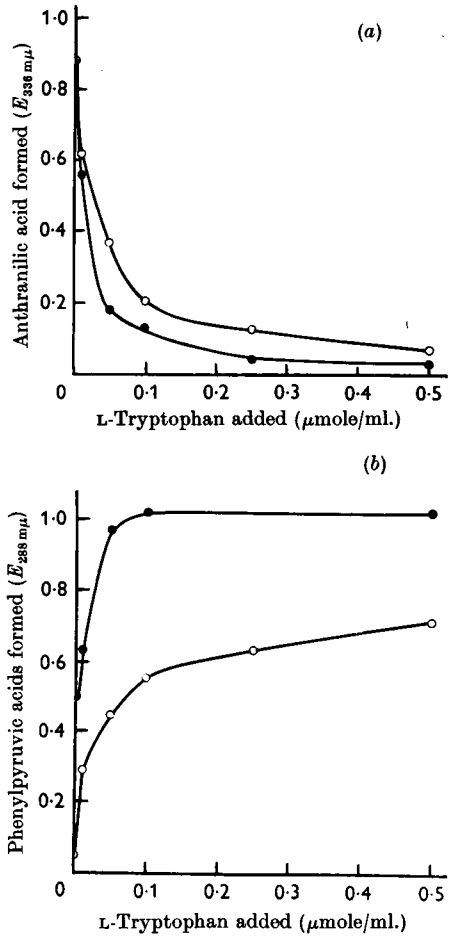


Fig. 8. Formation of phenylpyruvic acids as the result of inhibition of anthranilic acid synthesis by tryptophan. All tubes contained the complete reaction mixture (see Fig. 1) for anthranilic acid production with crude extract of strain T17 (1·7 mg. of protein/ml.) and L-tryptophan as indicated. After incubation for 45 min. at 37°, samples (1 ml.) were acidified and extracted with 4 ml. of ethyl acetate and spectra measured after 16 hr. at 30°. A parallel set of tubes was set up containing hydroxylamine (1 μmole/ml.). (*a*) Inhibition of anthranilic acid formation. (*b*) Increasing concentrations of phenylpyruvic acids formed with increasing inhibition of anthranilic acid synthesis. ○, Without hydroxylamine; ●, with hydroxylamine.

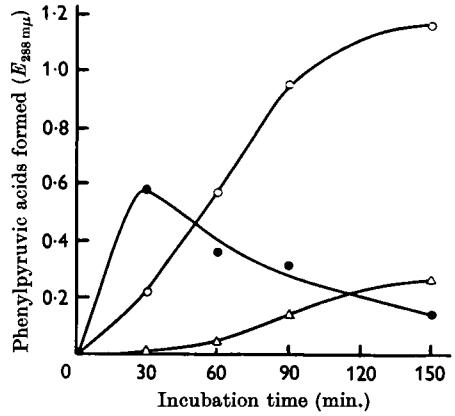


Fig. 9. Rate of appearance of phenylpyruvic acids at different concentrations of bacterial extract. Crude T17 extract (△, 0·46 mg. of protein/ml.; ○, 0·91 mg. of protein/ml.; ●, 1·82 mg. of protein/ml.) was incubated with the complete reaction mixture minus glutamine (see Fig. 1) at 37°. Experimental samples (0·5 ml.) were acidified and extracted with 4 ml. of ethyl acetate. Complete spectra of ethyl acetate layers were measured after storage for 16 hr. at 30°.

Table 8. *Requirements for the conversion of shikimic acid 5-phosphate into phenylpyruvic acids by 0–60 % ammonium sulphate-precipitated enzyme preparation*

All tubes contained 50 μmoles of tris–HCl buffer, pH 8·2, 5 μmoles of $MgCl_2$, 1 μmole of shikimic acid 5-phosphate, strain T 17 (0–60 % ammonium sulphate-precipitated) enzyme preparation (1·4 mg. of protein), in a final volume of 1 ml. Incubated for 90 min. at 37°. Phenylpyruvic acids were assayed by extraction of 1 ml. of acidified reaction mixture with 4 ml. of ethyl acetate. Complete spectra of the ethyl acetate solutions were recorded after 16 hr. at 30°.

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Ribose 5-phosphate (mM) | 3 | 3 | – | – | 3 |
| Phosphoenolpyruvic acid (mM) | – | – | 2 | 2 | 2 |
| NAD (mM) | 1 | 1 | – | – | 1 |
| $NADH_2$ (mM) | – | – | 1 | 1 | 1 |
| Crude extract boiled (3 mg. of protein) | – | + | – | + | – |
| Phenylpyruvic acids formed ($E_{288\,m\mu}$) | 0·005 | 0·580* | 0·020 | 0·689* | 0·680* |

* Characteristic spectrum present (see Fig. 7).

Table 9. *Effect of phosphoenolpyruvic acid, ribose 5-phosphate, reduced nicotinamide–adenine dinucleotide and nicotinamide–adenine dinucleotide on production of the phenylpyruvic acids by 0–60 % ammonium sulphate-precipitated enzyme preparation*

Details were as given for Table 8 except that the enzyme preparation (1·8 mg. of protein/tube) was obtained from strain NC3 (0–60 % ammonium sulphate-precipitated) enzyme preparation.

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Phosphoenolpyruvic acid (mM) | 2 | – | 2 | 2 | 2 |
| $NADH_2$ (mM) | 1 | 1 | – | – | 1 |
| NAD (mM) | – | – | – | 1 | – |
| Ribose 5-phosphate (mM) | 3 | 3 | 3 | 3 | – |
| Phenylpyruvic acid detected ($E_{288\,m\mu}$) | | | | | |
| Incubation for 30 min. | 0·56* | 0·052* | 0·0 | 0·02 | 0·404* |
| Incubation for 70 min. | 0·56* | 0·094* | 0·0 | 0·0 | 0·126* |

* Characteristic spectrum present (see Fig. 7).

Table 10. *Phosphorylated sugars as substrates for the production of phenylpyruvic acids by 0–60 % ammonium sulphate-precipitated enzyme preparation*

Details were as given for Table 8 except that the enzyme preparation (2·4 mg. of protein/tube) was obtained from strain NC3. Sugar phosphate was added (3 mM) as indicated. Phenylpyruvic acids were assayed after extraction of 0·5 ml. of acidified reaction mixture into 3 ml. of ethyl acetate. Spectra were recorded after 16 hr. at room temperature. The characteristic spectrum was present in all samples.

| | Phenylpyruvic acids ($E_{288\,m\mu}$) | |
|---|---|---|
| | After 20 min. | After 40 min. |
| Ribose 5-phosphate | 0·412 | 0·415 |
| Glucose 6-phosphate | 0·461 | 0·520 |
| Fructose 6-phosphate | 0·545 | 0·490 |
| No sugar phosphate | 0·269 | 0·110 |

for anthranilic acid production as given in Table 2 minus glutamine, with the addition of boiled crude extract (3 mg. of protein/ml.).

*Enzyme preparations fractionated between 30 and 40 % saturation with ammonium sulphate.* The enzyme that converts shikimic acid plus ATP into shikimic acid 5-phosphate was present largely in enzyme fractions precipitated between 30 and 40 % saturation with ammonium sulphate. Such fractions required phosphoenolpyruvic acid, $NADH_2$ and boiled extract for the conversion of shikimic acid 5-phosphate into the phenylpyruvic acids. Neither ribose 5-phosphate nor NAD was required for the conversion.

A 30–40 % ammonium sulphate-precipitated fraction was unable to convert shikimic acid 5-phosphate into the phenylpyruvic acids in a reaction mixture containing ribose 5-phosphate, NAD, magnesium chloride and boiled extract at pH 8·2. However, addition of 50–60 % fraction to such a system resulted in the production of the phenylpyruvic acids. This indicates that some of the enzymes involved in the production of phosphoenolpyruvic acid and $NADH_2$ from ribose 5-phosphate and NAD are in the 50–60 % fraction.

## DISCUSSION

These studies have confirmed and extended studies on anthranilic acid production briefly reported by Srinivasan (1959). Srinivasan obtained a crude cell extract of *E. coli* capable of converting shikimic acid 5-phosphate into anthranilic acid in

the presence of magnesium chloride and L-glutamine at pH 8·2. He found that L-glutamine was a better source of nitrogen for anthranilic acid than L-aspartic acid, L-glutamic acid, L-asparagine or ammonium chloride, and that NAD or NADP and yeast extract were sometimes required for anthranilic acid production. With crude cell extracts, the requirements of magnesium chloride, L-glutamine, NAD and pH 8·2 have been confirmed in the present studies. Srinivasan found that the yield of anthranilic acid from shikimic acid 5-phosphate was 86%, whereas the yield from shikimic acid plus ATP was only 18%. In contrast with this, our studies showed that phosphorylation of shikimic acid occurred very rapidly, and shikimic acid and shikimic acid 5-phosphate were equivalent as substrates. Ribose 5-phosphate, glucose 6-phosphate or fructose 6-phosphate stimulated shikimic acid 5-phosphate removal and anthranilic acid production.

While the experiments described above were in progress it was reported that *A. aerogenes* T17, although accumulating anthranilic acid, was probably blocked in the conversion of *N*-(5-phosphoribosyl)anthranilic acid into 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate (Doy, Rivera & Srinivasan, 1961). The former compound is labile and readily broken down to anthranilic acid. Under the experimental conditions described above anthranilic acid is formed by any tested strain of *E. coli* or *A. aerogenes* not blocked between shikimic acid and anthranilic acid. Further, many of the results quoted have been confirmed with *E. coli* W 2–5, a strain apparently blocked immediately after anthranilic acid (Doy *et al.* 1961).

If 3-enolpyruvylshikimic acid 5-phosphate is an intermediate in the formation of anthranilic acid this would explain why phosphoenolpyruvic acid stimulated anthranilic acid production with ammonium sulphate-precipitated enzyme preparations. The complete system for anthranilic acid production contained substrates that could presumably provide phosphoenolpyruvic acid. Known pathways of carbohydrate metabolism could provide from sugar phosphate, as well as phosphoenolpyruvic acid, a source of $NADH_2$ and ATP if catalytic amounts of ADP were present. This may explain therefore the fact that shikimic acid was sometimes converted into anthranilic acid in the absence of added ATP. The reason for the decrease in anthranilic acid production observed when excess of ATP or phosphoenolpyruvic acid was added to the system is not known. Since inorganic phosphate inhibits anthranilic acid production, the inhibitions by ATP and phosphoenolpyruvic acid may be due to the release of inorganic phosphate as a result of phosphatases present in the cell extracts. It is also possible that ATP competes for a substrate required for anthranilic acid production by reacting with it.

There is an obligatory requirement for $NADH_2$ for the production of both the phenylpyruvic acids and anthranilic acid by partially purified enzyme preparations. $NADH_2$ may be required for the formation of a common precursor for all these compounds. The general pathway of aromatic synthesis has been discussed in some detail by Gibson *et al.* (1962).

Sugar phosphate was usually still required in the presence of added $NADH_2$ and phosphoenolpyruvic acid. As $NADH_2$ was readily oxidized by both crude and ammonium sulphate-precipitated enzyme preparations the requirement may have been due to the necessity for a $NADH_2$-regenerating system.

The yield of anthranilic acid from shikimic acid in the complete system was never above about 80%, and was frequently lower. Fig. 8(*b*) shows that, when hydroxylamine was added to this system, the phenylpyruvic acids were detected in the reaction mixture. This indicates that probably under usual conditions the phenylpyruvic acids were formed in comparatively low yield, transaminated in the presence of glutamine to phenylalanine and tyrosine, and so not detected.

Studies on the production of phenylpyruvic acid and 4-hydroxyphenylpyruvic acid by crude and partially purified extracts have shown that the substrates required are identical with those required for anthranilic acid production, with the exception of glutamine. Experiments with *E. coli* have indicated that prephenic acid is the immediate precursor of the phenylpyruvic acids (Schwinck & Adams, 1959; Weiss, Gilvarg, Mingioli & Davis, 1954). Prephenic acid is unstable under acid conditions, forming phenylpyruvic acid. Since in these studies spectra were examined after acidification of reaction mixtures to pH $< 2$ it is probable that any prephenic acid present would have been converted into phenylpyruvic acid. Direct tests for prephenic acid in the reaction mixtures were negative.

It is known that a number of amino acids may repress the synthesis of enzymes connected with their own formation. De-repression of enzyme synthesis occurs when auxotrophs have stopped growing in the presence of limiting end product. Therefore cell extracts for these studies on anthranilic acid were prepared from tryptophan auxotrophs which had been harvested after the end of growth in limiting indole. The de-repression of enzyme synthesis that occurs when strain T17 is grown in the presence of limiting tryptophan has been described (Doy & Morgan, 1963). Extracts of wild-type cells grown in minimal medium have only approximately one-fifth of the activity of extracts

prepared from tryptophan auxotrophs grown in minimal medium plus limiting indole.

Omission of glutamine from an enzyme system capable of forming anthranilic acid from shikimic acid led to the formation of the phenylpyruvic acids. This finding confirmed observations with whole cells (Doy & Gibson, 1961), which suggested that the nitrogen of anthranilic acid is introduced in the first reaction specific to tryptophan biosynthesis. It was also found that the addition of L-tryptophan to an enzyme system capable of forming anthranilic acid diverted intermediates along the phenylalanine–tyrosine pathways. This provides further evidence that L-tryptophan inhibits the first reaction specific to tryptophan biosynthesis.

## SUMMARY

1. Studies have been carried out on the conversion of shikimic acid into anthranilic acid, a precursor of tryptophan, with crude cell extracts of *Aerobacter aerogenes* T17, a tryptophan auxotroph unable to convert anthranilic acid into indolylglycerol phosphate. Substrates required for maximum anthranilic acid production were ATP, magnesium chloride, L-glutamine, NAD or $NADH_2$ and a sugar phosphate (ribose 5-phosphate, glucose 6-phosphate or fructose 6-phosphate). Inorganic phosphate and L-tryptophan strongly inhibited anthranilic acid production. Phosphoenolpyruvic acid and excess of ATP were also inhibitory. Shikimic acid plus ATP could be replaced by shikimic acid 5-phosphate as a substrate.

2. Requirements for the conversion of shikimic acid 5-phosphate into anthranilic acid by ammonium sulphate-precipitated enzyme preparations were phosphoenolpyruvic acid, $NADH_2$, L-glutamine and phosphorylated sugar.

3. Omission of glutamine or addition of L-tryptophan to the system resulted in decrease of anthranilic acid production and stimulation of the production of phenylpyruvic acid and 4-hydroxyphenylpyruvic acid. The requirements for the production of the phenylpyruvic acids by crude extracts from shikimic acid were magnesium chloride, ATP, phosphorylated sugar and NAD. Inorganic phosphate was inhibitory.

4. Requirements for the conversion of shikimic acid 5-phosphate into the phenylpyruvic acids by ammonium sulphate-precipitated enzyme preparations were phosphoenolpyruvic acid, $NADH_2$, and magnesium chloride at pH 8·2. Boiled crude extract and phosphorylated sugar stimulated the conversion and were sometimes obligatory requirements for aged preparations.

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## REFERENCES

Bratton, A. C. & Marshall, E. K. (1939). *J. biol. Chem.* **128**, 537.
Davis, B. D. (1948). *J. Amer. chem. Soc.* **70**, 4267.
Davis, B. D. & Mingioli, E. S. (1953). *J. Bact.* **66**, 129.
Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1959). *Data for Biochemical Research*, p. 192. Oxford: Clarendon Press.
Doy, C. H. & Gibson, F. (1959). *Biochem. J.* **72**, 586.
Doy, C. H. & Gibson, F. (1961). *Biochim. biophys. Acta*, **50**, 495.
Doy, C. H. & Morgan, P. N. (1963). *J. gen. Microbiol.* (in the Press).
Doy, C. H., Rivera, A., jun. & Srinivasan, P. R. (1961). *Biochem. biophys. Res. Commun.* **4**, 83.
Gaitonde, M. K. & Gordon, M. W. (1958). *J. biol. Chem.* **230**, 1043.
Gibson, F. & McDougall, B. (1961). *Aust. J. exp. Biol. med. Sci.* **39**, 171.
Gibson, M. I., Gibson, F., Doy, C. H. & Morgan, P. N. (1962). *Nature, Lond.*, **195**, 1173.
Gots, J. S., Koh, W. Y. & Hunt, G. R. (1954). *J. gen. Microbiol.* **11**, 7.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Lederberg, J. & Zinder, N. (1948). *J. Amer. chem. Soc.* **70**, 4267.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Morgan, P. N., Gibson, M. I. & Gibson, F. (1962). *Nature, Lond.*, **194**, 1239.
Moyed, H. S. (1960). *J. biol. Chem.* **235**, 1098.
Nyc, J. F., Mitchell, H. K., Leifer, E. & Langham, W. H. (1949). *J. biol. Chem.* **179**, 783.
Pittard, A. J., Gibson, F. & Doy, C. H. (1961). *Biochim. biophys. Acta*, **49**, 485.
Pittard, A. J., Gibson, F. & Doy, C. H. (1962). *Biochim. biophys. Acta*, **57**, 290.
Rivera, A., jun. & Srinivasan, P. R. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 864.
Schwinck, I. & Adams, E. (1959). *Biochim. biophys. Acta*, **36**, 102.
Srinivasan, P. R. (1959). *J. Amer. chem. Soc.* **81**, 1771.
Tatum, E. L., Bonner, D. & Beadle, G. W. (1943). *Arch. Biochem.* **3**, 477.
Vogel, H. J. & Bonner, D. M. (1956). *Microb. Genet. Bull.* **13**, 43.
Weiss, W., Gilvarg, C., Mingioli, E. S. & Davis, B. D. (1954). *Science*, **119**, 774.
Yanofsky, C. (1955). *J. biol. Chem.* **217**, 345.