4. A quantitative comparison of the reactivity of sugars and of phenylglycosides with antibody was made by establishing the molar concentration giving 50% inhibition of precipitation in the corresponding antigen-antibody system. The differences so found are considered to be due to different degrees of structural dissimilarity between a hexose in the free state and in glycosidic linkage.

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Phosphorylation of Shikimic Acid by Ultrasonic Extracts of Micro-Organisms

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The role of shikimic acid (I) as a common precursor of the essential aromatic metabolites tyrosine, phenylalanine, anthranilic acid (the precursor of tryptophan), *p*-aminobenzoic acid and *p*-hydroxybenzoic acid in some micro-organisms has been established (see Davis, 1955). Extracts of a phenylalanine auxotroph of *Escherichia coli* have been shown by Levin & Sprinson (1960) to convert shikimic acid 5-phosphate (II), a compound accumulated in the growth medium by some multiple aromatic mutants that do not grow on shikimic acid (Davis & Mingioli, 1953; Weiss & Mingioli, 1956), into 3-enolpyruvylshikimic acid 5-phosphate (III); they suggested that this last compound is the active intermediate in the formation of prephenic acid (IV), the unstable precursor of phenylalanine and probably also of tyrosine (see Davis, 1958). The conversion of shikimic acid 5-phosphate in the presence of L-glutamine into

anthranilic acid (V) by extracts of a mutant of *E. coli* blocked in the utilization of this acid (Srinivasan, 1959), and into *p*-aminobenzoic acid (VI) by extracts of baker's yeast (Weiss & Srinivasan, 1959), has been demonstrated.

Shikimic acid 5-phosphate is thus apparently possessed of a multiple intermediary function and a possible metabolic sequence, based on structural considerations and known enzymic reactions, has been suggested by Sprinson (1960). This is illustrated in Fig. 1, X being a possible common intermediate of anthranilic acid, *p*-aminobenzoic acid and, perhaps, *p*-hydroxybenzoic acid (VII). However, as Sprinson (1960) has pointed out, the relationships that emerge on the basis of nutritional requirements and accumulation patterns of multiple aromatic auxotrophs are entirely different, although interpretation of these results may well be complicated by the existence of internal inhibition phenomena. Doy & Gibson (1961), as a result of a study of the formation of 4-hydroxyphenylpyruvic acid and phenylpyruvic acid by tryptophan

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mutants and prototrophs of *Aerobacter aerogenes*, have recently proposed the following pathway:

It is clear that shikimic acid 5-phosphate occupies a key position in the biosynthetic pathway. This paper is concerned with the synthesis of this compound by transphosphorylation from adenosine triphosphate to shikimic acid catalysed by ultrasonic extracts of various micro-organisms; the name 'shikimic acid kinase' is proposed for the responsible enzyme. A preliminary account of the work has been given (Fewster, 1959); E. B. Kalen (cited by Davis, 1958) has previously reported that shikimic acid is 'converted to its 5-phosphate through reaction with adenosine triphosphate'.

MATERIALS AND METHODS

Organisms. *Escherichia coli* 518 (prototroph) and the multiple aromatic auxotrophs *E. coli* 83-1 (blocked in the conversion of dehydroquinic acid into dehydroshikimic acid), *E. coli* 83-24 (blocked in the utilization of shikimic acid) and *Aerobacter aerogenes* A170-40 (which accumulates shikimic acid 5-phosphate) were maintained by monthly subculture on tryptic digest of meat-agar slopes stored at 4° after incubation for 18 hr. at 37°; the three auxotrophs

were the gift of Dr B. D. Davis. *Acetobacter suboxydans* A.T.C.C. 621 was maintained by monthly subculture on yeast extract-glycerol-agar slopes (36 hr. incubation at 30°) and the following as stab cultures in tryptose-glucose-agar: *Leuconostoc mesenteroides* L39 (24 hr., 30°), *Leuconostoc mesenteroides* P60 (24 hr., 37°) and *Lactobacillus arabinosus* 17-5 (24 hr., 37°). Suspensions of washed organisms were presented as follows: *Arthrobacter globiformis* N.C.I.B. 8602 (Dr J. G. Morris), *Corynebacterium erythrogenes* 142 (Dr R. G. Tucker) and *Pseudomonas ovalis* Chester (Dr H. L. Kornberg).

Media and growth conditions. The minimal medium of Davis & Mingioli (1951), to which glucose solution, sterilized separately, was added to a final concentration of 0.5%, was used for *E. coli* 518. For *E. coli* 83-1 the same medium with glucose (0.5%) was supplemented with either a sterile solution of shikimic acid (final concentration 0.5 mM) or a mixture of the following aromatic compounds: DL-tyrosine (0.5 mM), DL-phenylalanine (0.5 mM), DL-tryptophan (0.25 mM), *p*-aminobenzoic acid (0.7 μM), *p*-hydroxybenzoic acid (0.7 μM). Minimal medium supplemented with glucose and the aromatic mixture detailed above was used for *E. coli* 83-24. For the maximum accumulation of shikimic acid 5-phosphate, *A. aerogenes* A170-40 was grown in minimal medium (without citrate) supplemented with glucose and limiting amounts of the aromatic compounds according to the directions of Weiss & Mingioli (1956); for bulk growth of this same strain for the preparation of extracts the aromatic compounds were added at the higher concentrations detailed above. Erlenmeyer flasks (1 l.) containing 500 ml. of medium were inoculated from fresh slope cultures with about 10⁹ organisms and shaken (circular motion, 220 rev. of radius 2 cm./min.) at 37° for 16 hr.

Fig. 1. Metabolic sequence suggested by Sprinson (1960)

A. suboxydans was grown in the following medium: peptone (Evans Medical Supplies Ltd., Liverpool), 5 g.; yeast extract (Marmite Ltd., London), 5 g.; sorbitol, 90 g./l.; the pH was adjusted to 6.0 with *N*-NaOH and the medium sterilized by autoclaving for 15 min. at 115°. This medium (500 ml. in 2 l. Erlenmeyer flasks) was inoculated with about 10⁹ organisms from a fresh slope culture and incubated for 72 hr. at 30° on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 200 rev. of radius 1.75 cm./min.

Ln. mesenteroides L39, *Ln. mesenteroides* P60 and *Lb. arabinosus* 17-5 were grown in the following medium: Evans peptone, 20 g.; Bacto yeast extract (Difco Laboratories Inc., Detroit, Mich., U.S.A.), 5 g.; Oxoid liver extract (Oxo Ltd., London), 1.6 g.; K₂HPO₄, 5 g.; salts B, 5 ml.; glucose 20 g./l. (Salts B contained, per l.: MgSO₄, 7H₂O, 40 g.; NaCl, 2 g.; FeSO₄, 7H₂O, 2 g.; MnSO₄, 4H₂O, 2 g.; 10*N*-HCl, 2 ml.) The pH was adjusted to 6.8 with *N*-NaOH and the medium sterilized by autoclaving for 10 min. at 115°. Erlenmeyer flasks (800 ml.) containing 500 ml. of medium were inoculated with 0.5 ml. of a 24 hr. culture of the organisms in tryptose-glucose broth and incubated for 18 hr. at the optimum growth temperature.

Assessment of growth and mass of organisms. The bacterial dry weight of cultures and suspensions was assessed with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) in 6 mm. tubes and with a neutral density filter and a separate calibration curve used for each of the organisms.

Preparation of extracts. Organisms were harvested by centrifuging, washed once with half the culture volume of 0.9% (w/v) NaCl and then with water, and finally suspended in water at a concentration of about 80 mg. dry wt./ml. Samples (10–20 ml.) in a 50 ml. stainless-steel conical flask were subjected to ultrasonic vibrations with a Mullard ultrasonic generator Type E. 7590/B (Mullard Electronic Equipment, London); the vibrations (25 kcyc./sec.) were generated by a magnetostrictor (1 in. stack) brazed to the base of the flask, the exterior of which was cooled with running water so that its contents remained at less than 20° throughout. For the disruption of organisms of the strains of *Ln. mesenteroides*, *Lb. arabinosus* and *Arthrobacter globiformis* the suspensions were exposed to the vibrations for 13 min.; suspensions of the other organisms were treated for 5 min. Whole organisms and cellular debris were removed by centrifuging (10 000*g* for 20 min. at 0°) and the supernatants stored at –15°; transphosphorylative activity from ATP to shikimic acid was retained after several weeks at this temperature.

Purification of extracts of E. coli 518. The crude extract obtained as described above was centrifuged at 100 000*g* for 1 hr. at 0° in a Spinco model L ultracentrifuge; all phosphorylative activity towards shikimic acid was retained in the supernatant. For removal of metal ions, samples of this supernatant (about 40 mg. of protein/ml.) were dialysed against 5 mm-EDTA in 20 mm-phosphate buffer (all phosphate buffers used were prepared from stock solutions of Na₂HPO₄·12H₂O and KH₂PO₄), pH 6.5, for 15 hr. and finally against 20 mm-phosphate buffer, pH 6.5, for 3 hr., all operations being carried out at 0–4°. To remove nucleic acid from the supernatant obtained by high-speed centrifuging, samples (final concentration 10–12 mg. of protein/ml.) in 0.2*M*-phosphate buffer, pH 6.5, were treated with increasing amounts of a solution of protamine sulphate

(10 mg./ml. in 0.2*M*-phosphate buffer, pH 6.5) and, after standing for 10 min., were centrifuged, all at 0°. Protein concentration, nucleic acid concentration and phosphorylative activity of the supernatants were measured (see the Results and Discussion section); nucleic acid precipitation was maximal (88%) at a final concentration of protamine sulphate of 2 mg./ml., representing 0.18 mg. of protamine sulphate/mg. of protein. Fractionation of samples freed of nucleic acid was carried out in 0.2*M*-phosphate buffer, pH 7.0, by the addition of solid (NH₄)₂SO₄ at 0°; 100% saturation was taken as complete saturation at room temperature, not at 0°, and the amount of solid to be added to obtain a given degree of saturation was determined from the nomograms of Dixon (1953). Phosphorylative activity of the precipitates, recovered by centrifuging and dissolved in 0.1*M*-phosphate buffer, pH 7.0, was measured. In the fraction precipitated between 30 and 50% saturation, 85% of the original absolute activity was recovered associated with 48% of the original protein. Attempts to purify this precipitate further by refractionation with (NH₄)₂SO₄ led to almost complete loss of activity.

Analytical methods. For the determination of transphosphorylative activity in crude extracts of the various micro-organisms the following incubation mixture was used: bacterial extract, 10–20 mg. of protein; shikimic acid, 10 μmoles (0.2 ml. of a neutralized 50 mm-shikimic acid solution); ATP, 20 μmoles (0.2 ml. of a neutralized 0.1*M*-solution of the disodium salt of ATP); MgCl₂, 20 μmoles; KF, 30 μmoles; phosphate buffer, pH 6.5, 150 μmoles; total volume, 3.0 ml.; pH 6.5 was shown to be the optimum pH for the activity of crude extracts of *E. coli* 518. The reaction was started by the addition of bacterial extract (warmed to the incubation temperature) to the warmed mixture of the other components in upright tubes (5 in. × $\frac{5}{8}$ in.) and the mixture incubated for up to 2 hr. at the optimum temperature for growth of the organism under investigation. Phosphorylation was determined by measuring the disappearance of free shikimic acid. After incubation the reaction mixtures were deproteinized and the phosphate esters precipitated by the addition to each tube of 1.0 ml. of 0.3*N*-Ba(OH)₂ and 1.0 ml. of 5% (w/v) ZnSO₄ (Somogyi, 1945). The mixtures were centrifuged and the shikimic acid content of diluted samples of the supernatants relative to those of controls from which (a) bacterial extract and (b) ATP were omitted was determined by the method of Gaitonde & Gordon (1958) with a Unicam SP. 600 spectrophotometer. Enzymic activity is expressed as μm-moles of shikimic acid disappearing/mg. of protein/hr.

ATP consumption as well as shikimic acid disappearance was determined in incubation mixtures with the supernatant obtained by high-speed centrifuging of crude extracts of *E. coli* 518; ATP was measured as acid-labile phosphate, by the method of Berenblum & Chain (1938) for the estimation of the inorganic phosphate released. Tris-maleate buffer (Gomori, 1948), pH 7.0, was used in place of phosphate in the incubation mixtures and the reaction was stopped and mixtures were deproteinized by the addition of trichloroacetic acid solution to a final concentration of 6% (w/v), followed by centrifuging.

Protein concentration in crude bacterial extracts was determined by a method based on the biuret reaction (Gornall, Bardawill & David, 1949). Protein in extracts that had been treated with protamine sulphate or (NH₄)₂SO₄

or both was measured spectrophotometrically according to O. Warburg and W. Christian (cited by Layne, 1957); the ratio of absorption at 280 m μ and at 260 m μ also gave information on the relative amount of nucleic acid present.

Chromatography. Shikimic acid and shikimic acid 5-phosphate were separated chromatographically on paper with either of these developing solvent mixtures: butan-1-ol-acetic acid-water (100:6:25, by vol.) or phenol-water (3:1, v/v) containing 1.0% (w/v) of formic acid. The compounds were rendered visible by spraying with the reagents described by Cartwright & Roberts (1955) for quinic acid. Incubation mixtures were deproteinized by the addition of trichloroacetic acid (6%, w/v, final concentration) and, after centrifuging, 0.1 ml. of the supernatant was taken for chromatographic analysis.

Chemicals. Shikimic acid was supplied by the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; ATP and tris (Sigma Chemical Co., Mo., U.S.A.), and protamine sulphate (from herring roe; L. Light and Co. Ltd., Colnbrook, Bucks.) were also commercial products.

RESULTS AND DISCUSSION

Phosphorylation of shikimic acid by various strains of Escherichia coli

Crude extracts. Crude extracts of *E. coli* 518 (prototroph) catalysed the phosphorylation of shikimic acid at a rate of 120–140 μ m-moles/mg. of protein/hr. at the optimum pH of 6.5. Similar values were obtained for extracts of *E. coli* 83-1 regardless of whether the organisms were harvested from minimal medium supplemented with shikimic acid or an excess of the complete aromatic mixture, i.e. enzyme formation was not suppressed by the presence in the growth medium of an excess of the ultimate products. Extracts of *E. coli* 83-24, a mutant reported by Davis & Mingioli (1953) to accumulate mainly shikimic acid and only traces of shikimic acid phosphate in its culture medium, possessed phosphorylative activity of the same order as that of the prototroph. These results are summarized in Table 1.

Partially purified extracts. The activity of the supernatant obtained by high-speed centrifuging of crude extracts of *E. coli* 518 was 280–320 μ m-moles/mg. of protein/hr. at the optimum pH of 7.0; Fig. 2 is a plot of phosphorylative activity against concentration of the supernatant in terms of protein. The pellet derived from the centrifuging was completely devoid of activity; thus shikimic acid kinase, like the enzymes responsible for the phosphorylation of carbohydrates in micro-organisms, is not associated with a particulate fraction. The decrease in shikimic acid concentration catalysed by the supernatant was accompanied by an equivalent decrease in ATP concentration (see Table 2). Paper-chromatographic analysis of deproteinized reaction mixtures showed the presence of a compound that gave a yellow colour

after treatment with the reagents described by Cartwright & Roberts (1955) and with R_f identical with that of shikimic acid 5-phosphate that accumulated in the growth medium of *A. aerogenes* A170-40 when grown in the presence of limiting amounts of the aromatic mixture.

Table 1. *Phosphorylation of shikimic acid by crude extracts of various micro-organisms*

Incubation mixture was as described under 'Analytical methods'; tubes were incubated for 1.5 hr. at the optimum growth temperature of the respective micro-organisms.

Organism	Activity (μ m-moles of shikimate disappearing/mg. of protein/hr.)
<i>Escherichia coli</i> 518*	135
<i>Escherichia coli</i> 83-1†	140
<i>Escherichia coli</i> 83-1‡	130
<i>Escherichia coli</i> 83-24‡	120
<i>Acetobacter suboxydans</i> 621	80
<i>Aerobacter aerogenes</i> A170-40	165
<i>Arthrobacter globiformis</i> 8602	130
<i>Corynebacterium erythrogenes</i> 142	160
<i>Pseudomonas ovalis</i> Chester	60

* Grown in minimal medium.

† Grown in minimal medium supplemented with shikimic acid.

‡ Grown in minimal medium supplemented with the aromatic mixture.

Fig. 2. Plot of phosphorylative activity at pH 7.0 against protein concentration of the supernatant obtained by high-speed centrifuging of a crude extract of *E. coli* 518; tubes were incubated for 1.5 hr. at 37°.

Heating of samples of the supernatant in 0.1 M-phosphate buffer, pH 7.0, for 5 min. at 50° caused a 65% loss in activity; total loss resulted after 5 min. at 55°.

After dialysis of samples of the supernatant against EDTA complete abolition of phosphorylative activity was obtained by exclusion of Mg^{2+} ions from the incubation mixture; full activity was restored by the addition of Mg^{2+} or Mn^{2+} ions to a final concentration of 6.6 mM. In this requirement for bivalent-metal ions shikimic acid kinase resembles other phosphate-transferring

Table 2. Utilization of shikimic acid and adenosine triphosphate by extracts of *Escherichia coli*

Minimal incubation mixture: supernatant obtained by high-speed centrifuging of a crude extract of *E. coli* 518, 31 mg. of protein; $MgCl_2$, 30 μ moles; KF, 50 μ moles; tris-maleate buffer, pH 7.0, 150 μ moles. Final total volume, 3.0 ml. Tubes were incubated for 1.5 hr. at 37°.

Incubation mixture	Shikimate utilized (μ moles)	ATP utilized (μ moles)
Minimal + shikimate (20 μ moles)	0.4	—
Minimal + ATP (20 μ moles)	—	4.5
Complete [minimal + shikimate (20 μ moles) + ATP (20 μ moles)]	9.0	13.0
Complete less control values	8.6	8.5

Fig. 3. Fractionation of the supernatant (final concentration 11.25 mg. of protein/ml. of 0.2 M-phosphate buffer, pH 6.5), obtained by high-speed centrifuging of a crude extract of *E. coli* 518, with protamine sulphate; ▲, specific activity; △, absolute activity; ●, protein concentration; ○, nucleic acid concentration of the supernatants.

enzymes. The phosphorylation reaction was not inhibited by 0.2 mM-phenylmercuric acetate or by any of the final aromatic products of the biosynthetic pathway at mM concentration. With 3 mM-iodoacetate 55% inhibition was obtained. Phosphorylation by the supernatant was not increased by anaerobic conditions.

The results of the titration of samples of the supernatant in 0.2 M-phosphate buffer, pH 6.5, with protamine sulphate are illustrated in Fig. 3, the plots shown being the mean values obtained from three experiments. The addition of protamine sulphate to a final concentration of 2 mg./ml., representing 0.18 mg. of protamine sulphate/mg. of protein, gave, after centrifuging, a supernatant containing 68% of the original protein and only 12% of the original nucleic acid; higher concentrations of protamine sulphate did not further reduce the nucleic acid content. This supernatant retained 94% of the original phosphorylative activity (i.e. activity/unit volume), corresponding to an increase of 38% in the specific activity (i.e. activity/mg. of protein). By treatment of this supernatant freed of nucleic acid with solid ammonium sulphate 85% of the absolute activity of it was recovered in the fraction precipitated between 30 and 50% saturation associated with 48% of the original protein; the specific activity of this precipitate was 710 and represents a fivefold purification of the original crude extract. Attempts to purify this precipitate further by refractionation with ammonium sulphate led to almost complete loss of activity.

Phosphorylation of shikimic acid by other micro-organisms

Table 1 records the phosphorylative activity towards shikimic acid of crude ultrasonic extracts of a number of micro-organisms known to be capable of synthesizing aromatic amino acids. These values do not differ greatly from those obtained for the various strains of *E. coli* (Table 1). The heavy accumulation (500–1000 mg./l.) of shikimic acid 5-phosphate in growing cultures of *Aerobacter aerogenes* A170-40 (Weiss & Mingioli, 1956) suggests that the shikimic acid kinase of this micro-organism is not inhibited by the product of its action. Such an inhibition might be postulated to explain the accumulation of mainly shikimic acid in cultures of *E. coli* 83-24, organisms of which have been shown to contain an active shikimic acid kinase.

No phosphorylative activity could be measured in ultrasonic extracts of *Ln. mesenteroides* L39 and *Ln. mesenteroides* P60, micro-organisms recognized as being incapable of synthesizing aromatic amino acids.

No activity was detected in extracts of *Lb. arabinosus* 17-5 grown in complex medium. Ory &

Lyman (1955) showed that shikimic acid could replace glucose and a gas phase containing carbon dioxide as a requirement for the synthesis of tyrosine and phenylalanine by this micro-organism (Lyman, Moseley, Wood, Butler & Hale, 1947). Atkinson & Fox (1951) reported the isolation of stable variants of *Lb. arabinosus* 17-5 able to synthesize aromatic amino acids. If phosphorylation of shikimic acid is indeed an obligatory reaction when conditions are such that the synthesis of aromatic amino acids is possible by this micro-organism it may be that the enzyme responsible is adaptive. However, from the work described with the mutant *E. coli* 83-1 (which is unable to synthesize shikimic acid) it appears that the shikimic acid kinase of *E. coli* is constitutive. An alternative explanation for the absence of activity from the extracts is that the enzyme is sensitive to ultrasonic vibrations, although this is obviously not so with *Arthrobacter globiformis* (see Table 1), an organism which also required the longer exposure time (13 min.) for disruption.

SUMMARY

1. A method for determining the capability of ultrasonic extracts of micro-organisms of catalysing transphosphorylation from adenosine triphosphate to shikimic acid is described.

2. The responsible enzyme, to which the name shikimic acid kinase is given, is present in mutants and wild-type *Escherichia coli*. It is soluble, has an optimum pH of 7.0, exhibits a requirement for Mg^{2+} or Mn^{2+} ions, is not inhibited by 0.2 mM-phenylmercuric acetate, and is 55% inhibited by 3 mM-iodoacetate. Formation of the enzyme is not suppressed and its activity is not inhibited by the ultimate products of the aromatic biosynthetic pathway.

3. Enzyme activity is measured by determination of the rate of disappearance of shikimic acid and of adenosine triphosphate from the incubation mixture. The product of the reaction is shown to be chromatographically identical with shikimic acid 5-phosphate accumulated in the growth medium of *Aerobacter aerogenes* A170-40 when grown in the

presence of limiting amounts of the full aromatic supplement. Fivefold purification of the enzyme is described.

4. The presence of shikimic acid kinase in extracts of a number of micro-organisms capable of synthesizing aromatic amino acids is reported. The enzyme is shown to be absent from extracts of some micro-organisms recognized as being incapable of synthesizing these acids.

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