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Rapid report

Transport of the antibacterial agent (6*S*)-6-fluoroshikimate and other shikimate analogues by the shikimate transport system of *Escherichia coli*

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Abstract

We show that the antibacterial agent, (6*S*)-6-fluoroshikimate, is a substrate for the shikimate transport system of *Escherichia coli* because in exchange–diffusion experiments it displaced intracellular [^{14}C]shikimate with the same kinetics as did unlabelled shikimate. Other shikimate analogues were also substrates: as judged by similar experiments or, in the case of (6*R*)-6-fluoroshikimate, by inference.

Keywords: Fluoroshikimate; *shiA*; Shikimate transport; Antibacterial agent

(6*S*)-6-Fluoroshikimate inhibits the growth of *Escherichia coli* on a glucose-ammonium-salts medium: most likely by being converted to fluorochorismate and subsequently inhibiting the synthesis of *p*-aminobenzoate [1,2]. However, spontaneous resistant variants occur at high frequency, which is predicted to limit the usefulness of the compound for antibacterial chemotherapy in human medicine [3]. Such resistant variants cannot take up [^{14}C]shikimate whereas wild-type strains can; and in *E. coli* K-12, the resistance mapped at or near *shiA* [3], one of at least two loci that determine the shikimic acid uptake phenotype [4,5].

The specificity of the shikimic acid transport system is unexplored: but because of the above observations we predicted that (6*S*)-6-fluoroshikimate should be a substrate. Here we describe the results of testing this hypothesis, using an exchange–diffusion method devised by Brown and Doy [5]. We explored the specificity of the shikimate transport system of *E. coli* further by performing similar experiments with other shikimate analogues.

Fig. 1 shows the structures of shikimic acid and its analogues used in the present work. The analogues **II**, **III** and **IV** were prepared substantially pure at ZENECA Pharmaceuticals. Compounds **V**, **VI** and **VII** were provided by E. Haslam (Sheffield University) as an unresolved mixture in molar proportion 68:24:8, respectively. Shikimic acid, barium chorismate, amino acids, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were from Sigma, Dorset, UK. [^{14}C]Shikimic acid (21.9 mCi/mmol; 100 μCi/ml) was from New England Nuclear. Other reagents were obtained from commercial sources at analytical grade.

For transport experiments, bacteria were grown in liquid, or on agar-solidified, Medium 56 [6] containing glucose (0.2%, w/v), and the following supplements: shikimic acid (10 μg/ml), thiamine (3.4 μg/ml), and proline, arginine and histidine (25 μg of each /ml). *E. coli* strain AB1360 [*his4 proA argE aroD xyl thi lac gal mtl tsx* (λ)$^-$ F$^-$] was obtained from A.J. Pittard (University of Melbourne, Parkville, Victoria, Australia). Strain AB1360-7 was selected by us as a spontaneous variant that would take up shikimic acid more rapidly than the parent strain, AB1360 [3]. The selection was carried out as described by Brown and Doy [5], by picking rapidly-growing variants from agar plates of Medium 56 agar [6] containing shikimic acid (100 μg/ml) as the sole source of aromatic compounds. The identity of the variant was confirmed by its possession of the following auxotrophic markers of the

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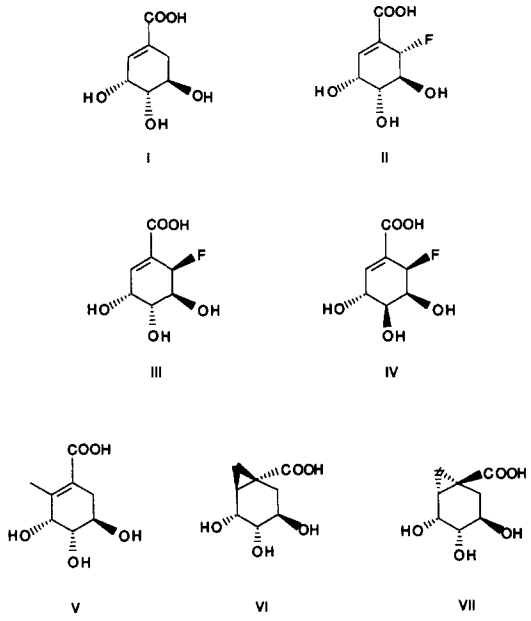

Fig. 1. The structures of shikimic acid and its analogues studied in the present work. **I**, Shikimic acid; **II**, (6*S*)-6-fluoroshikimic acid; **III**, (6*R*)-6-fluoroshikimic acid; **IV**, (6*R*)-6-fluoro-4-epihydroxyshikimic acid; **V**, 2-methylshikimic acid; **VI**, 1,2-β-methyleneshikimic acid; **VII**, 1,2-α-methyleneshikimic acid.

parent strain: *his*, *pro*, *arg*, *aro* and *thi*. Susceptibility-testing was performed using *E. coli* K-12 NCTC 10538 as described previously [3]: using 2-ml portions of solidified Davis and Mingioli glucose-salts medium supplemented with thiamine.

In order to demonstrate exchange–diffusion, we adopted the conditions used by Brown and Doy [5]; that is: low density of cells and large sample volume; and a low initial concentration of the reporter substrate, [^{14}C]shikimate, followed by a thousand-fold excess of the non-radiolabelled test substrate. We chose not simply to measure inhibition of the uptake of [^{14}C]shikimate because this would not have differentiated between competitive binding of a test substrate without its transport, and competitive transport per se.

A starter culture was grown without shaking in 50 ml of Medium 56 in a 100-ml conical flask at 37°C for 16 h, to an OD_{550} of about 0.85 (here and below, optical densities refer to a path length of 1 cm). Two further 50-ml volumes of the same medium were inoculated with 0.5 ml each of the starter culture. These were incubated without shaking at 37°C for 8–10 h, whereupon the OD_{550} was 0.30–0.33. The suspensions were combined and the cells harvested by centrifugation at $7800 \times g$ for 10 min at 30°C (Sorvall RC5C; SS-34 rotor: R_{av} 7 cm) and washed twice by resuspension, in fresh Medium 56 containing glucose but none of the auxotrophy supplements, and recentrifugation as above. The final pellet was resuspended in 10 ml of Medium 56 containing glucose and the proline, histidine and thiamine supplements. Arginine was omitted in order to reduce the amount of protein synthesis that would occur during the uptake experiments. The cells were then kept at 37°C for 30 min, without shaking, in order to exhaust any remaining shikimate or aromatic biosynthetic intermediates. The OD_{550} was then 1.61–1.82 (0.62–0.75 mg dry mass/ml). A 0.1-ml volume of this suspension was added to 4.9 ml of pre-warmed supplemented Medium 56 (but without arginine) in a 25-ml conical flask, and the new suspension maintained at 37°C for 3 min without shaking. At zero time, [^{14}C]shikimic acid was added to a final concentration of 1.52 μM (16.7 μl of a ten-fold dilution in water of the stock solution described above) and the flask shaken at 37°C. At specified times, samples were handled in one of two ways, depending on whether total cell-associated radiolabel, or only radiolabel incorporated into trichloroacetic acid (TCA)-insoluble material, was being measured. Where the total cell-associated radiolabel was being measured, a preliminary 0.1-ml sample was taken shortly before the full sample time point, in order to measure empirically the cpm per unit volume of the reaction mixture. Beckman Ready Value aqueous scintillation fluid (4 ml) was added to this sample and the mixture counted as described below. The remaining suspension in the flask was vacuum-filtered at the specified time through a 0.45-μm pore size, type HA Millipore filter. The flask was rinsed with 20 ml of 'wash-buffer' held at 37°C (50 mM Hepes/150 mM NaCl, that had been adjusted to pH 7.5 using 10 M NaOH solution), and the rinse drawn through the same filter. The filter was then washed with two more 20-ml volumes of the same wash-buffer. Where TCA-insoluble radiolabel was being measured, samples were handled as follows. At the required time, 20 ml of ice-cold TCA solution (5%, w/v) was mixed rapidly with the 5 ml contents of the flask. The mixture was kept ice-cold for 15 min. A 0.1-ml liquid sample was then taken and mixed with 4.0 ml of scintillation fluid for determining empirically the specific radioactivity of the reaction mixture. The remaining sample of 24.9 ml was vacuum-filtered as above. The flask was rinsed and the filter washed also as described above, but this time with 20-ml volumes of ice-cold 5% TCA. For both types of determination, filters were dried at room temperature, placed in scintillation minivials and 4 ml of scintillation fluid added. All samples were counted in a Beckman LS 3801 counter and the counts corrected for background before analysis. The TCA-soluble pool of radiolabel was calculated as the difference between the total cell-associated [^{14}C]shikimate and the TCA-insoluble fraction.

For some of the flasks, a further addition was made 60 s after the addition of [^{14}C]shikimic acid. This consisted of either a 50-μl or a 100-μl volume of a concentrated solution of unlabelled compound whose ability to elicit exchange–diffusion was being studied. The solutions were as follows (see Fig. 1): shikimic acid, **I**, 17.4 mg/ml to a final concentration of 0.99 mM; **II**, 19.2 mg/ml to a final concentration of 0.99 mM, compound **IV**, 9.6 mg/ml to a

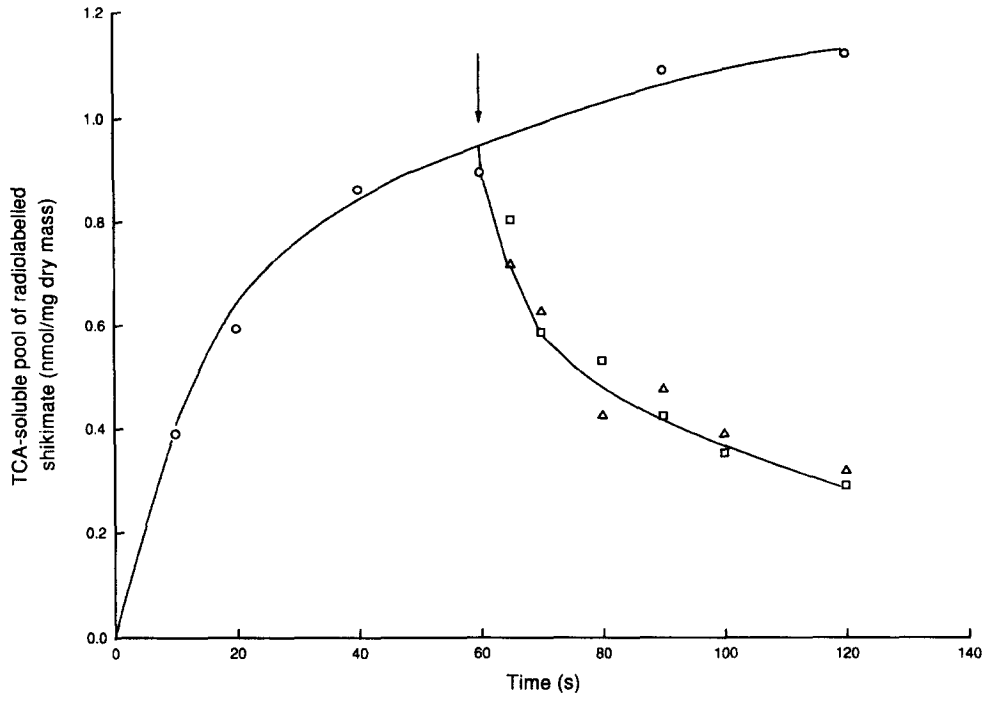

Fig. 2. Uptake of [^{14}C]shikimate into the TCA-soluble pool of *E. coli*, and its displacement by excess unlabelled shikimate or (6*S*)-6-fluoroshikimate. (○) Uptake of [^{14}C]shikimate at 1.5 μM; (□) addition of unlabelled shikimate to 0.99 mM at 60 s (arrow); (△) addition of (6*S*)-6-fluoroshikimate **II** to 0.99 mM at 60 s.

final concentration of 0.98 mM; the mixture of **V**, **VI** and **VII**, 9.4 mg/ml to final concentrations of 0.67, 0.24 and 0.08 mM, respectively; and barium chorismate, 18 mg/ml to a final concentration of 0.98 mM. Apart from these additions, samples were handled, and the radioactivity counted as described above.

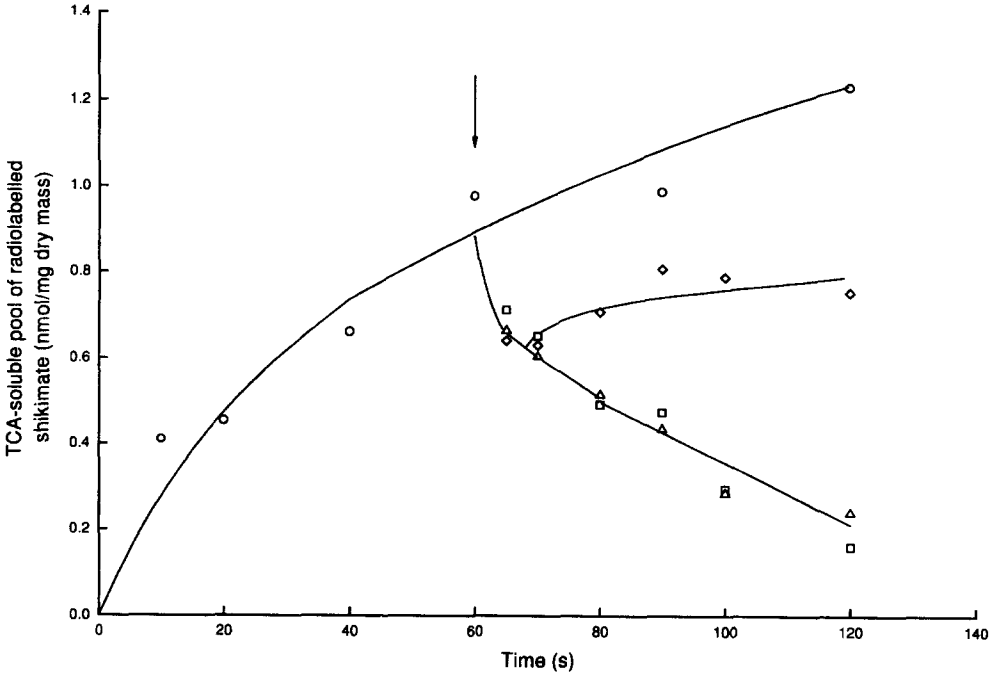

Fig. 3. Uptake of [^{14}C]shikimate into the TCA-soluble pool of *E. coli*, and the effects of adding excess unlabelled shikimate, a mixture of three shikimate analogues, or chorismate. (○) Uptake of [^{14}C]shikimate at 1.5 μM; (□) addition of unlabelled shikimate to 0.99 mM at 60 s (arrow); (△) addition of the mixture of **V**, **VI** and **VII** to 0.67, 0.24 and 0.08 mM, respectively, at 60 s; (◇) addition of chorismate to 0.98 mM at 60 s.

The uptake of [^{14}C]shikimate into the TCA soluble pool of *E. coli* AB1360-7 was about three times more rapid than uptake into the parent strain, AB1360; and the size of the cell-associated TCA-soluble pool was larger by a factor of 4 (data not shown). This increased pool size allowed us to perform the desired exchange–diffusion experiments [5].

The addition of a 650-fold excess (0.99 mM) of unlabelled shikimic acid **I** or of (6*S*)-6-fluoroshikimic acid **II** caused kinetically-identical outflows of the pool of TCA-soluble ^{14}C (Fig. 2). A mixture of three other shikimic acid analogues: 2-methylshikimic acid **V**, 1,2-β-methylene-shikimic acid **VI** and 1,2-α-methylene-shikimic acid **VII**, at a combined final concentration of 0.98 mM, but in the ratio 68:24:8, respectively, also elicited an outflow with identical kinetics to that caused by shikimic acid (Fig. 3). One further shikimic acid analogue was tested, (6*R*)-6-fluoro-4-epihydroxyshikimic acid **IV**. When it was added at a final concentration of 0.98 mM, it similarly caused an outflow of TCA-soluble radiolabel indistinguishable from that caused by shikimic acid itself (data not shown). Chorismate at a final concentration of ca. 1 mM did not cause the same outflow of radiolabelled shikimate (Fig. 3). The addition of either carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at a final concentration of 20 μM, or a mixture of cyanide and azide at a final concentration of 5 mM of each, caused uptake to halt, but there was no outflow of radiolabel similar to that displayed in Figs. 2 and 3 (data not shown). The addition of toluene at a final concentration of 2% (w/v) caused a complete loss of the radiolabelled TCA-soluble intracellular pool of shikimate within 5 s (data not shown).

The energetics of shikimate transport have not been studied previously. One reason why outflow was not observed when the uncoupler or respiration inhibitors were added might be that the transport system is kinetically 'gated' and can only operate in the presence of a trans-cytoplasmic-membrane protonmotive force or difference in electric potential. Tobramycin transport in *E. coli*, for example, only operates in the presence of a transmembrane electric potential of greater magnitude than about -120 mV [7], which could account for some previously-unexplained observations on the kinetics and energetics of aminoglycoside transport in general [8,9].

The hypothesis tested in the experiments reported here, namely that (6*S*)-6-fluoroshikimate **II** is a substrate for the shikimate transport system of *E. coli*, has been shown to be correct. This confirms that the variants that were resistant to (6*S*)-6-fluoroshikimate, and which also failed to take up shikimate [3], were resistant due to a lack of transport of the antibacterial compound into the cytoplasm.

The shikimate transport system appeared to have a fairly broad specificity. Compound **IV** (Fig. 1), was also a substrate of the shikimate transporter indistinguishable from shikimate itself. The mixture of **V**, **VI** and **VII** behaved indistinguishably from shikimate (Fig. 3); but we cannot conclude whether these three compounds were recognised equally by the shikimate transport system, because only the mixture was available to us. Lastly, we infer that the transport system can accept one further analogue as a substrate, (6*R*)-6-fluoroshikimate **III**, even though we did not have enough compound to perform exchange–diffusion experiments. The rationale for this inference is as follows. We have previously reported that in liquid Davis and Mingioli medium, using a low number of cells for the inoculum, *E. coli* K-12 was susceptible to both (6*S*)-6-fluoroshikimate (IC_{50}, 0.031 μg/ml) and (6*R*)-6-fluoroshikimate (IC_{50}, 8.0 μg/ml) [3]. The single-step, spontaneous (6*S*)-6-fluoroshikimate-resistant variant derived from this strain of *E. coli* K-12, 'variant 20' (IC_{50} of (6*S*)-6-fluoroshikimate, 512 μg/ml), was resistant due to lack of shikimate transport activity (see [3] for details). In the same susceptibility-test experiment, the IC_{50} of (6*R*)-6-fluoroshikimate for variant 20 was also 512 μg/ml [3]. In other words, the absence of shikimate transport activity in variant 20 conferred on the strain resistance to both (6*S*)-6-fluoroshikimate and (6*R*)-6-fluoroshikimate. From this we conclude that the latter molecule must also be a substrate of the transport system.

From the results obtained with shikimate analogues **II**, **IV**, **V**, **VI** and **VII** (three of these being added as an unresolved mixture, Fig. 3), and deducing that (6*R*)-6-fluoroshikimate **III** is a substrate, the transport system appears to tolerate a variety of structural features at positions 1, 2, 4 and 6 of the shikimate molecule. The most surprising of these is perhaps the acceptance of the epimeric hydroxyl at C-4 in **IV**.

One more experiment is relevant to this observation. We determined the susceptibility of *E. coli* K-12 to **IV** at the same time as determining its susceptibility to **II** and **III**. The 2-ml agar-incorporation method [3] yielded MICs (minimum inhibitory concentrations) of **II**, **III** and **IV** of 0.125, 64 and >1024 μg/ml, respectively. Incidentally, the higher MICs of **II** and **III** than those reported previously [3] were due to the different method used: i.e. agar-solidified medium with a higher inoculum cell-density. Thus **IV** was not antibacterial despite being a substrate for the shikimate transporter, and predictedly entering the bacterial cytoplasm. We suggest that this is because **IV**, or one of its metabolites, was not a substrate of one of the enzymes further along the shikimate pathway; for example, shikimate kinase or EPSP (enolpyruvylshikimate-3-phosphate) synthase. This would be consistent with the idea that the orientation of the hydroxyl at C-4 in several intermediates of the pathway is important in substrate binding [10,11]; and that this orientation should be the same as that in shikimate itself for the 6-fluoro derivatives to be recognised as good substrates by the later enzymes of the pathway.

As a practical consequence of the findings that a variety of shikimate analogues are accepted as transport substrates, one might attempt to exploit the broad specificity of the

shikimate transport system to smuggle other toxic shikimate analogues into bacterial cells as a means of designing antibacterial agents. However, because the system also appears to be lost at high frequency [3]; if analogues of shikimate are to be successful antibacterial agents, it will be necessary to introduce them into the bacterial cytoplasm via one or several other transport systems in addition to that which mediates the uptake of shikimate itself (e.g. see [12]).

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