5. The enzyme was inhibited by *p*-chloro-mercuribenzoate with reversal of inhibition by cysteine. Iodoacetate was slightly inhibitory.

The authors wish to express their gratitude to Professor T. A. Bennet-Clark, in whose Department this research was carried out. One of us (D.B.) wishes to acknowledge with thanks the award of a King's College Tutorial Studentship and subsequently of a Witwatersrand Council of Education Scholarship.

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2. MODE OF ATTACHMENT OF SHIKIMIC ACID AND DEHYDROSHIKIMIC ACID TO DEHYDROSHIKIMIC REDUCTASE

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The extraction of dehydroshikimic reductase from etiolated epicotyls of pea seedlings and the purification of the enzyme have been described by Balinsky & Davies (1961). In order to determine the mode of attachment of the substrates shikimic acid and dehydroshikimic acid to the enzyme, various compounds of similar structure to these substrates were examined for possible competitive inhibition. A preliminary report of this work has already been published (Balinsky & Davies, 1960).

EXPERIMENTAL

Special chemicals. Shikimic acid was obtained from L. Light and Co. Ltd. Triphosphopyridine nucleotide was obtained from the Sigma Chemical Co.

Enzyme preparation. A purified enzyme preparation was obtained as described by Balinsky & Davies (1961).

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Measurement of enzyme activity. The enzyme was assayed as described by Balinsky & Davies (1961), except that the shikimic acid concentration was altered as indicated in Tables 1 and 2 and in Figs. 1-3. In addition, the compound being examined for inhibition was added at the concentrations shown in the Tables.

Some difficulty was experienced when testing pyrogallol, catechol, resorcinol, gallic acid and resorcylic acid. These compounds are readily oxidized by atmospheric or dissolved oxygen, and the increase in extinction at 340 m μ due to this oxidation masked any increase due to enzyme activity. In order to overcome this, all solutions were prepared in oxygen-free water and covered with a layer of toluene. Buffer, enzyme and substrates were pipetted into the cuvette and a few drops of toluene were added before addition of the polyphenol or hydroxy acid. Under these conditions, there was only a very slight blank reaction due to the added compound.

RESULTS

Several of the compounds tested were inhibitory (Table 1). Some of these compounds were more extensively studied to determine whether inhibi-

tion was competitive or non-competitive and to evaluate the inhibitor constants. The concentrations of inhibitor were kept constant and the shikimic acid concentration was varied as shown in Figs. 1-3. Plots were made of the reciprocal of the reaction velocity ($1/v$) against the reciprocal of the shikimic acid concentration ($1/[S]$) (Lineweaver & Burk, 1934) at a constant concentration of enzyme. The lines meet in a point on the ordinate axis, indicating competitive inhibition (Friedenwald & Maengwyn-Davies, 1954).

Table 1. *Effect of various compounds on enzyme activity*

Quartz cuvettes of 1 cm. light-path contained 0.1M-glycine-NaOH buffer, pH 9.0, 0.17 mM-triphosphopyridine nucleotide, purified enzyme, sodium shikimate [mM in series (A) and 0.3 mM in series (B)] and the compound under examination (neutralized) at the concentration shown. The reaction was assayed by measuring production of reduced triphosphopyridine nucleotide at 340 $m\mu$ in a Unicam SP. 500 spectrophotometer.

Compound added	Concentration (mM)	Activity (% of original)
(A) None	—	100
Quinic acid	10	100
Dehydroquinic acid	10	100
Salicylic acid	10	100
2:4-Dihydroxybenzoic acid	10	97
Benzoic acid	10	100
Phenol	10	97
Pyrogallol	10	0
Tryptophan	10	97
(B) None	—	100
Catechol	5	0
Guaiacol	5	9
Vanillin	0.5	0
Galic acid	5	0
Protocatechuic acid	1	77
<i>p</i> -Hydroxybenzoic acid	5	0
<i>m</i> -Hydroxybenzoic acid	5	100
<i>p</i> -Aminobenzoic acid	5	100
3:5-Dihydroxybenzoic acid	5	100

Table 2. *Inhibitor constants for competitive inhibitors of dehydroshikimic reductase*

Quartz cuvettes of 1 cm. light-path contained 0.1M-glycine-NaOH buffer, pH 9.0, 0.17 mM-triphosphopyridine nucleotide, purified enzyme, shikimic acid and inhibitor at the concentrations shown in Figs. 1-3. The reaction was followed at 340 $m\mu$ in a Unicam SP. 500 spectrophotometer. Inhibitor constants were calculated from the slopes of the lines obtained in Figs. 1-3.

Inhibitor	Inhibitor constant (mM)
Vanillin	0.093
Galic acid	0.38
<i>p</i> -Hydroxybenzoic acid	0.74
Protocatechuic acid	0.75
Catechol	1.9
Guaiacol	11.0

Table 2 shows that vanillin and the hydroxy acids are potent inhibitors, catechol is somewhat weaker and guaiacol is weaker still.

DISCUSSION

Those compounds which inhibited the reaction were found to do so competitively, presumably by occupying the site normally serving for the attachment of shikimic acid and dehydroshikimic acid. A comparison of the structures of these inhibitors with those of the natural substrates might give an indication of the groups required for attachment of the substrate to the enzyme. The inhibitors all possess at least two groups in the same positions as those occupied in shikimic acid, one of these being a hydroxyl group corresponding to the 4-OH position of the shikimic acid and dehydroshikimic acid molecules. Substituents in the benzene ring lie in the plane of the ring, and it will

Fig. 1. Lineweaver-Burk plots for shikimic acid in the presence and absence of inhibitor. Quartz cuvettes of 1 cm. light-path contained 0.1M-glycine-NaOH buffer, pH 9.0, 0.17 mM-triphosphopyridine nucleotide, purified enzyme, shikimic acid at the concentrations shown: O, without inhibitor; ●, with 0.093 mM-protocatechuic acid. The reaction was followed at 340 $m\mu$ in a Unicam SP. 500 spectrophotometer. v , $\Delta E_{340}/\text{min}$.

be shown below that the 4-OH of shikimic acid is approximately in the plane of the ring.

Shikimic acid (I) has a conformation which is partly planar and partly alicyclic. A 'half-chair' conformation with substituents in the equatorial plane is the most stable for *cyclohexene* compounds

(Klyne, 1954; Barton & Cookson, 1956). There are two possible 'half-chair' conformations of shikimic acid (II and III; equatorial and quasi-equatorial bonds are indicated by horizontal lines). It will be seen that structure (II) has only one hydroxyl group in the equatorial plane, whereas in structure (III) both the 3- and 4-OH groups are equatorial,

Fig. 2. Lineweaver-Burk plots for shikimic acid in the presence and absence of inhibitor. Conditions of assay were as described in Fig. 1. $\bigcirc$, No inhibitor; $\bullet$, 2 mM-catechol; $\bigcirc$, 0.5 mM-gallic acid. v , $\Delta E_{340}/\text{min}$.

Fig. 3. Lineweaver-Burk plots for shikimic acid in the presence and absence of inhibitor. Conditions of assay were as described in Fig. 1. $\bigcirc$, No inhibitor; $\bullet$, 2 mM-guaiacol; $\bigcirc$, 55 μM -vanillin; $\bigcirc$, mM-*p*-hydroxybenzoic acid. v , $\Delta E_{340}/\text{min}$.

hence this structure is more stable. There is also a possibility of hydrogen-bond formation, not only between the *cis* 4- and 5-OH groups, but also between the equatorial 3- and 4-OH groups in (III), which would further increase the stability of this conformation. It should be noted that these hydroxyl groups are alcoholic in nature, not

phenolic as with the aromatic inhibitors. The aromatic hydroxyl groups would be partially ionized at the pH of the assay.

In dehydroshikimic acid, all carbon atoms except C-3 are coplanar, and the 3- and 4-OH groups are still approximately equatorial. The presence of an equatorial 4-OH group, both in the aromatic inhibitors observed and in the natural substrates, suggests the possible role of this group in the attachment of the substrate to the enzyme. Since phenol is not an inhibitor, it appears that at least one other group, corresponding to the carboxyl or 3- or 5-OH of shikimic acid, must be present for attachment. The nature of the other group seems to be less specific. Individual examples will be discussed below.

Gallic acid is the aromatic inhibitor most closely related in structure to shikimic acid. Besides attachment in the 4-OH position, attachment might also occur at the carboxyl and 3-OH groups. It is less likely to occur at the 5-OH position, since the corresponding hydroxyl group of shikimic acid is not equatorial, and is absent in dehydroshikimic acid. But a planar substituent in the 5-position should not cause steric hindrance, since dehydroshikimic acid itself has a planar substituent (the carbonyl oxygen) in this position. Similarly, in protocatechuic acid attachment in the 3-OH position is possible. But inhibition by *p*-hydroxybenzoic acid indicates that attachment of a 3-OH group is not essential. All three acids are strong inhibitors (Table 2).

In pyrogallol and catechol, two adjacent hydroxyl groups in the absence of a carboxyl group appear to be sufficient, although catechol is a less potent inhibitor than the hydroxy acids described above. From this it can be concluded that either a 1-carboxyl or 3-OH substituent is necessary for enzyme attachment, in addition to the 4-OH group, but that the attachment at the carboxyl position is more conducive to stability of the enzyme-inhibitor complex. In shikimic acid and dehydroshikimic acid, all three positions may be utilized.

Inhibition by guaiacol and vanillin presents another interesting aspect. In guaiacol, the attachment apparently takes place at the methoxyl group in place of the hydroxyl group of catechol, though inhibition is weak. Strong inhibition by vanillin indicates that, in addition to rather weak attachment at the methoxyl group, there may be fairly firm attachment of the aldehyde group at the position normally occupied by the carboxyl group. It appears that the requirements for attaching groups in the 1- and 3-positions are not entirely specific.

Among the non-inhibitors, *p*-aminobenzoic acid indicates that a 4-amino group cannot replace the

4-OH group. Salicylic acid, *m*-hydroxybenzoic acid, 3:5-dihydroxybenzoic acid and resorcinol possess no groups in the same positions relative to the 4-OH group as shikimic acid. On the other hand, if the carboxyl groups of salicylic acid, *m*-hydroxybenzoic acid and resorcylic acid are assumed to occupy the position of the carboxyl group of shikimic acid, there is no hydroxyl group corresponding to the 4-position of the ring. 2:4-Dihydroxybenzoic acid, however, might be expected to inhibit, since it differs from *p*-hydroxybenzoic acid only in the possession of a 2-OH group. The lack of observed inhibition is presumably due to steric hindrance in the 2- and 6-positions. Similarly, the observation that quinic acid (hexahydro-1:3:4:5-tetrahydroxybenzoic acid) and 5-dehydroquinic acid do not inhibit is probably due to steric hindrance, caused by the 1-OH group.

It appears therefore that a hydroxyl group in the position normally occupied by the 4-OH group of shikimic acid and dehydroshikimic acid is necessary for enzyme attachment, in addition to groups which can occupy the 1- and 3-positions of the ring and have similar properties with respect to attachment as the carboxyl and hydroxyl groups respectively. Substituents in the 2- and 6-positions, and non-equatorial substituents in the 1-position, apparently encounter steric hindrance. It is suggested that the natural substrates themselves are probably attached to the enzyme by means of the carboxyl and 3- and 4-OH groups. The possibility of direct attachment to the ring cannot be

Fig. 4. Diagrammatic representation of the suggested mode of attachment of shikimic acid to dehydroshikimic reductase. Solid semi-circles indicate proposed attachment sites. Possible steric hindrance to substituents in the 2- and 6-positions of the ring is also indicated. TPN⁺, Triphosphopyridine nucleotide.

ruled out. Fig. 4 is a diagram of the possible attachment sites (drawn in solid lines) on the enzyme surface.

SUMMARY

1. A number of aromatic and hydro-aromatic compounds of similar structure to the natural substrates of dehydroshikimic reductase, shikimic acid and dehydroshikimic acid, were examined for possible inhibition.

2. Several of the compounds found to cause inhibition were examined further, and were shown to inhibit competitively.

3. The inhibitor constants for these competitive inhibitors were calculated.

4. Since these compounds presumably inhibit by occupying the site normally serving for attachment of shikimic acid and dehydroshikimic acid, a theory of the mode of attachment of the substrates to the enzyme is discussed.

5. It is suggested that shikimic acid and dehydroshikimic acid are attached to the enzyme by means of the carboxyl and 3- and 4-hydroxyl groups.

The authors wish to express their gratitude to Professor T. A. Bennet-Clark, in whose Department this research was carried out. Our thanks are due to Dr L. Crombie for helpful discussions on the conformation of shikimic acid. One of us (D.B.) wishes to acknowledge with thanks the award of a King's College Tutorial Studentship and subsequently of a Witwatersrand Council of Education Scholarship.

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3. PREPARATION AND PROPERTIES OF DEHYDROQUINASE

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The preparation and purification of dehydroshikimic reductase, the enzyme converting dehydroshikimic acid into shikimic acid, was described by Balinsky & Davies (1961*a*). The present

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paper describes the preparation and purification of dehydroquinase, the enzyme catalysing the inter-conversion of dehydroquinic acid (I) and dehydroshikimic acid (II), as shown below.

A preliminary account has been published (Balinsky & Davies, 1960).

The presence of this enzyme was first demonstrated in cell-free extracts of *Escherichia coli* by Davis & Weiss (1953). It was purified eightfold by