A MICROCHEMICAL METHOD FOR THE DETECTION
AND DETERMINATION OF SHIKIMIC ACID*

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In certain microorganisms, shikimic acid has been shown to be a precursor for the biosynthesis of aromatic amino acids (1). Tracer experiments with cell-free extracts of Escherichia coli have revealed that shikimic acid is formed from uniformly labeled glucose (2). In view of the reports (3) of the biosynthesis of aromatic amino acids from glucose in infant mouse brain, an investigation was begun to determine whether central nervous tissue synthesizes shikimic acid or some analogous compound from glucose. The present report describes some observations on the oxidation of shikimic acid by periodic acid, and the application of this reaction to the quantitative determination of shikimic acid.

**Methods**

Oxidation of Shikimic Acid with Periodic Acid—Shikimic acid is oxidized by periodic acid to give trans-aconitic acid and a dialdehyde (4). Since trans-aconitic acid can be determined spectrophotometrically, after treatment with acetic anhydride and pyridine (5), a possible method for measurement of shikimic acid was suggested. Preliminary efforts to adapt this method to the quantitative determination of the oxidation products of shikimic acid were unsuccessful because of the presence of the excess periodic acid. The excess periodic acid was, therefore, removed by the addition of a saturated solution of barium hydroxide. It was noted that, when such a solution is allowed to stand, an intense yellow color develops. Investigations showed that this yellow color is not due to some breakdown product or derivative of periodic acid formed in the presence of barium hydroxide, but required the presence of certain oxidizable compounds in the reaction mixture. Later it was found that in the above reaction barium hydroxide could be conveniently replaced by sodium hydroxide. Of the many biologically important compounds tested only shikimic acid, quinic acid, and tryptophan produce a yellow color under alkaline conditions, after treatment with periodic acid. The formation of the yellow color from

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shikimic acid, which may be characteristic of the dialdehyde formed, was made the basis of the analytical method to be described.

**Paper Chromatography of Shikimic Acid and Other Related Compounds**—Shikimic acid may be detected on paper chromatograms (Whatman No. 1) by its absorption of ultraviolet light or by a spray reagent. The following two solvent systems were used: (1) acetic acid-ethyl acetate-water (10:30:30, top layer, and (2) acetic acid-n-butanol-water (10:40:50, top layer). Amounts of less than 20 μg are not detectable in ultraviolet light, but the spray reagent reveals quantities of the order of 5 μg of shikimic acid. For this purpose papers were dried by air and then heated in an oven (60–70°C) for ½ hour and sprayed with 0.1 per cent periodic acid, followed by a spray with benzidine reagent B, prepared according to Cifonelli and Smith (6). Shikimic acid and other compounds that are oxidized by periodic acid are revealed as white spots against a blue background of the chromatogram. In addition to the carbohydrates and their phosphate esters, the following compounds can be detected with this spray reagent: quinic acid, gallic acid, cysteine, methionine, ascorbic acid, glyoxal, inositol, cytidine, ATP, TPN, and DPN. If the chromatograms are allowed to age overnight, the white spots given by hexoses turn yellow and those given by pentoses turn brown.

**EXPERIMENTAL**

**Determination of Shikimic Acid**—To the test solution (3 ml.) containing 2 to 10 μg of shikimic acid, 0.5 ml. of a 1 per cent solution of periodic acid (HIO₄·2H₂O) is added, with mixing. After 3 hours the solution is made alkaline by the addition of 0.5 ml. of 1 N NaOH, mixed, and 0.3 ml. of 0.1 M glycine is immediately added to stabilize the color. The contents of the tube are mixed and the optical density of the solution at 380 μm is measured within 10 minutes after the addition of NaOH. All solutions must be filtered before use. Stock solutions of periodic acid and glycine give reproducible results in this reaction for a period of 2 weeks if they are stored in the cold.

**Effects of Various Factors on Optical Density of Oxidized Shikimic Acid Solution**

**Spectrum of Reaction Mixture**—The alkaline reaction mixture has a maximal absorption at 380 μm (Fig. 1) and a molar extinction coefficient of $4.76 \times 10^4$ (calculated as shikimic acid).

**Effect of pH**—The optical density of the reaction products of shikimic

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1 The following abbreviations are used: ATP, adenosine triphosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; TPP, thiamine pyrophosphate; TCA, trichloroacetic acid.
acid (10 γ) was measured at 380 mμ at several pH values in the alkaline range. There is little or no absorption below pH 9.0. An increment in optical density is observed at pH 9.5 and the optimal value is reached between pH 11 and 12.

**Amount of Periodic Acid**—The absorption obtained from a known amount of shikimic acid (0.19 × 10⁻⁴ M) increases as the molar concentration of periodic acid is changed from 15 × 10⁻⁴ to 73 × 10⁻⁴ M. In this concentration interval the optical density increased some 60 to 90 per cent. There is no further increment in the absorption with additional quantities of periodic acid (Fig. 2).

**Effect of Glycine**—The yellow color developed after addition of sodium hydroxide to the oxidized shikimic acid is not very stable. The fall in absorption, which is approximately linear during the first 10 to 15 minutes, is so rapid (Fig. 3, Curve A) that the method cannot be employed for quantitative determinations unless the time factor is rigidly controlled. Attempts to stabilize the optical density by changing the pH of the solution were not successful. However, addition of glycine (0.1 M) to the alkaline solution stabilized the color and gave a 12 per cent increment in the initial optical density.
density (Fig. 3, Curve B). This enabled samples to be measured within 10 minutes after color development with a reproducibility of 3 to 4 percent. The quantities of glycine greater than those recommended, or addition of glycine before the addition of alkali, reduces the absorbance of the solution considerably. In the latter case the absorbance is reduced by 55 per cent.

**Rate of Oxidation of Shikimic Acid with Periodic Acid**—Periodic acid was allowed to react with shikimic acid (10 μ) at room temperature for different time intervals, and the extent of oxidation of shikimic acid was determined according to the procedure described above. Under these conditions some 3 hours are required for the maximal reaction (Fig. 4).

**Beer's Law**—The absorbance of the reaction mixture is proportional to an original concentration of 1 to 7 μ per ml. of shikimic acid (Fig. 5). With larger amounts of shikimic acid it was found convenient to dilute to concentrations in this range. Under the conditions described it is possible to detect the oxidation products of shikimic acid at concentrations as low as 0.3 μ of shikimic acid per ml. of solution.

**Specificity of Reaction**—The structural analogy of quinic and gallic acids to shikimic acid suggested that these compounds might also react in a manner similar to shikimic acid. Gallic acid gives an immediate yellowish brown color, which darkens upon addition of NaOH. Quinic acid produces a pale yellow color only upon the addition of alkali; the absorption peak
is at 380 mμ, and the maximal absorption is reached 20 to 25 minutes after
the addition of NaOH. During this period the molar extinction coeffi-
cient (for quinic acid), which is 0.33 × 10^4 at zero time, increases to 1.65 ×
10^4. When the presence of quinic acid in solutions is suspected, shikimic
acid can be determined with a reproducibility of 6 to 7 per cent by measur-
ing the optical density of the solution immediately after the addition of

![Diagram](image.png)

**Fig. 4.** Curve showing the optimal time required for the maximal reaction of
shikimic acid with periodic acid, as measured from the optical density of the oxida-
tion products at 380 mμ. Conditions as described in Fig. 1, except as indicated in
the above figure.

**Fig. 5.** Curve showing the proportionality between the concentration of shikimic
acid and the optical density of the oxidation products at 380 mμ. Conditions as
described in Fig. 1.

NaOH and glycine, before significant color production from the quinic
acid.

The following series of compounds were tested according to the procedure
described at 1 mg. levels: (a) the common amino acids, glycocyanine,
glutathione, glucosamine, and histamine; (b) arabinose, xylose, ribose,
fructose, glucose, galactose, lactose, maltose, and sedoheptulose, and the
phosphate esters of these sugars; (c) citric, oxalacetic, malic, succinic,

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2 Aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, tryp-
tophan, valine, methionine, phenylalanine, leucine, isoleucine, glutamine, histidine,
lysine, arginine, proline, hydroxyproline, cyst(e)ine, homocyst(e)ine, ornithine,
citrulline, taurine.
α-ketoglutaric, lactic, and cis- and trans-aconitic acids; (d) adenine, guanine, thymine, cytosine, uracil, thymidine, cytidine, uric acid, orotic acid, nicotinic acid, ATP, TPN, DPN, and TPP; (e) glycine, adrenaline, and inositol. Of these, only those listed in Table I had any measurable effect on the reaction. The absorbance obtained from 10 γ of most of these compounds was negligible.

In the presence of glutathione and adrenaline (10 γ), only 42 to 46 per cent of the added shikimic acid can be accounted for. Ascorbic acid (1 mg.) completely inhibited the reaction and no shikimic acid is detectable in the solution. Gallic acid (10 γ) reduces the absorbance by 10 per cent, whereas quinic acid (10 γ) and tryptophan (10 γ) increase it by 5 to 12 per cent. Compounds like TPN, DPN, and TPP give a positive reaction but do not inhibit the reaction with shikimic acid, and 10 γ of most of the other compounds examined increased the optical density of the solution by 6 to 7 per cent in the determination of shikimic acid.

**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Addition of HIO₄</th>
<th>Action of NaOH on oxidized solution</th>
<th>Optical density of 10 γ at 380 mp per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shikimic acid</td>
<td>No color</td>
<td>Yellow</td>
<td>100</td>
</tr>
<tr>
<td>Quinic acid</td>
<td>&quot; &quot;</td>
<td>Dark brown</td>
<td>7</td>
</tr>
<tr>
<td>Gallic &quot;</td>
<td>Yellowish brown</td>
<td>Yellow</td>
<td>3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Gradual pale yellow</td>
<td>Brown</td>
<td>2</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>Yellowish → pink</td>
<td>Color disappears</td>
<td>Nil</td>
</tr>
<tr>
<td>Methionine</td>
<td>Gradual pale yellow</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>&quot; &quot; Immediate yellow</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>&quot; &quot; Immediate yellow</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Cysteine</td>
<td>&quot; &quot; Immediate yellow</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Cystine</td>
<td>Gradual pale yellow</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Homocystine</td>
<td>&quot; &quot; Immediate yellow</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>TPN</td>
<td>No color</td>
<td>Intense yellow</td>
<td>2</td>
</tr>
<tr>
<td>DPN</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
<td>4</td>
</tr>
<tr>
<td>TPP</td>
<td>&quot; &quot;</td>
<td>Pale yellow</td>
<td>6</td>
</tr>
</tbody>
</table>

Application of Method to Tissue Extracts

As TCA (10 per cent) was used for the precipitation of the tissue proteins, preliminary experiments were carried out to note the effect of TCA on the recovery of shikimic acid from an aqueous solution. Only 13 per cent of the added shikimic acid can be accounted for in the presence of TCA. When TCA was removed by four separate ether extractions of the acid
solution, the shikimic acid reacted quantitatively. Hence in all subsequent experiments in which TCA was used as a protein precipitant, the tissue extracts were washed with ether.

Protein-free filtrates of rat tissues were analyzed for shikimic acid. However, only fractions of added shikimic acid could be accounted for in the presence of such tissue extracts.

The interfering substances in the tissue extracts could not be removed by use of cation or anion exchange resins or by heat treatment of the extracts. It is not probable that ascorbic acid in the tissue extract can be the main cause of the low reactivity of shikimic acid because the ascorbic acid content of the tissues tested is below the limits which inhibit the reaction in pure solution.

However, application of paper chromatographic methods gave quantitative recoveries of shikimic acid added to tissue extracts. Of the solvent systems tested, only the acetic acid-ethyl acetate-water system, which separates shikimic acid ($R_f$ 0.52) from quinic acid ($R_f$ 0.36), and ascorbic acid ($R_f$ 0.46), gave good recoveries. The following procedure was finally adopted for the determination of shikimic acid in tissue extracts.

The ether-washed TCA extract (0.5 gm. of tissue) is heated for 1 hour in a boiling water bath and filtered if necessary. The clear filtrate is passed through a Permutit Q (H form) column. The effluent together with water washings are collected and taken to dryness in vacuo. The residue is

### Table II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Direct measurement on decationized tissue extract</th>
<th>After paper chromatography of tissue extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shikimic acid equivalent of tissue extract</td>
<td>Shikimic acid equivalent of tissue extract</td>
</tr>
<tr>
<td></td>
<td>(A)</td>
<td>(C)</td>
</tr>
<tr>
<td></td>
<td>$\gamma$ per gm. tissue</td>
<td>per cent</td>
</tr>
<tr>
<td>Brain</td>
<td>11.7</td>
<td>60</td>
</tr>
<tr>
<td>Liver</td>
<td>6.9</td>
<td>16</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.5</td>
<td>65</td>
</tr>
<tr>
<td>Muscle</td>
<td>3.8</td>
<td>69</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.1</td>
<td>68</td>
</tr>
<tr>
<td>Lung</td>
<td>2.0</td>
<td>69</td>
</tr>
<tr>
<td>Heart (per ml.)</td>
<td>2.3</td>
<td>49</td>
</tr>
<tr>
<td>Blood (per ml.)</td>
<td>1.1</td>
<td>67</td>
</tr>
</tbody>
</table>

The results given in Column A are corrected for tissue blanks (no periodic acid added).
transferred on to the Whatman No. 1 paper for chromatography with the acetic acid-acetate system.

After development of the chromatograms (about 18 inches), the papers were dried and the position of shikimic acid was determined by spraying a guide strip. Comparable areas on companion strips were eluted with water, the eluates were centrifuged, and an aliquot of the supernatant solution was treated according to the usual procedure. The results of a typical experiment given in Table II show that the recoveries of shikimic acid are low when the method is applied to deca tionized tissue filtrates directly; liver extracts give the lowest recovery (16 per cent). However, paper chromatography removes the interfering substances from the extracts of the tissues tested with the exception of those from liver and muscle. Tissue extracts treated (as above) give some absorption at 380 m\textmu; the values in Column A are these absorption values converted to shikimic acid equivalents. The lower values obtained in Column C undoubtedly reflect the removal of various substances (e.g. TPN and DPN) by chromatography, which react in the test. In Column B are recoveries of shikimic acid added to ether-washed TCA extracts of tissues, and in Column D are recoveries from such extracts after paper chromatography.

It appears from these results (Column C) that there are small amounts of substances in tissue extracts which have the same \( R_F \) as that of shikimic acid, and which give the shikimic acid test. The nature of these substances is under study.

SUMMARY

A method has been described for the detection and quantitative determination of shikimic acid. Under suitable conditions the method can be used for the determination of quinic acid also.

The method is not applicable to tissue extracts directly. However, paper chromatography separates various interfering compounds from shikimic acid. Elution of the shikimic acid from paper chromatograms permits its quantitative determination, by the method described, in all tissues with the exception of liver and muscle.

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