BIOSYNTHESIS OF TRYPTOPHAN AND GRAMINE IN YOUNG BARLEY SHOOTS

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Abstract—Shikimic acid-G-C" (i.e. generally labelled), anthranilic acid-G-C¹⁴, indole-2-C¹⁴, serine-3-C¹⁴ and indolylactic acid-G-C¹⁴ were found to be good precursors of bound tryptophan in young barley shoots. It is likely that tryptophan is formed in barley by the same route established for Escherichia coli.¹ ²

Tryptophan was readily converted to gramine in agreement with the prior observations of Marion et al.¹⁰⁻¹² and compounds converted readily to tryptophan were fairly good precursors of gramine. Serine-3-C¹⁴ gave gramine with about twice as much C¹⁴ in the methyl groups as in the rest of the molecule, whereas gramine formed from indole-2-C¹⁴ or anthranilic acid-G-C¹⁴ was not labelled in the methyl groups. Indolylacetic acid, indolylacetonitrile, indolylacetaldehyde, indolylacrylic acid and tryptamine were not readily converted to either tryptophan or gramine. Gramine was not converted back to tryptophan. None of the compounds tested appeared to be intermediates in the formation of gramine from tryptophan.

INTRODUCTION

The intermediate steps in the biosynthesis of tryptophan in Escherichia coli are known in considerable detail due to studies with biochemical mutants by Davis et al.¹⁻³ and, more recently, by Yanofsky and co-workers.⁴⁻⁵ A simplified scheme for tryptophan synthesis by E. coli is:

Carbohydrate —→ shikimic acid —→ anthranilic acid —→
indolylglycerol phosphate L-serine L-tryptophan + triose phosphate.

Indole can be substituted for indolylglycerol phosphate in this sequence,⁴⁻⁵ and indeed this substitution is necessary for in vivo experiments since indolylglycerol phosphate does not readily penetrate living cells.⁶

There is some evidence that the enzyme catalyzing the last step, tryptophan synthetase, is present in plants. Thus, Kretovich and Polyanovskii⁷⁻⁷ have shown that unlabelled compounds such as indole, anthranilic acid, serine and indolylpyruvic acid will cause an increase in the level of free tryptophan when they are administered to pea or wheat seedlings. Greenberg and Galston⁸ have presented evidence that pea seedling extracts contain trypto-

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⁷ V. L. Kretovich and O. L. Polyanovskii, Biokhimya 24, 917 (1959) (in English translation by Consultants Bureau Enterprises, Inc.).
Biosynthesis of tryptophan and gramine in young barley shoots

Phan synthetase, and similar results have been obtained by Holmsen and Teas with maize. These observations support the view that tryptophan is formed in plants as shown above, but to obtain more reliable evidence, it is desirable to use C\textsuperscript{14}-labelled precursors, since a net synthesis in such a complex system might be due to an indirect effect. The results of feeding labelled compounds to barley shoots are reported in the present paper.

Barley shoots were chosen as the experimental material because they also form gramine, a simple indole alkaloid that can be readily isolated. Marion et al. have shown that barley seedlings will convert tryptophan to gramine without rearrangement of the indole ring or loss of the ring-attached carbon (i.e. the $\beta$-carbon of tryptophan). Thus any good precursor of tryptophan should also be a fairly good precursor of gramine. In the present investigation this was tested by isolating both gramine and bound tryptophan from each sample of barley shoots after feeding a labelled precursor. This procedure gives a check on the conversion of various labelled compounds to tryptophan.

RESULTS AND DISCUSSION

The results shown in Table 1 support the view that tryptophan synthesis in barley follows the same pathway as in bacteria. This can be seen from the relatively low dilutions of C\textsuperscript{14} during incorporation of shikimic acid, anthranilic acid, indole or serine into tryptophan. The facile conversion of 3-indolyllactic acid to tryptophan is analogous to the conversions of phenyllactic acid to phenylalanine and of $p$-hydroxyphenyllactic acid to tyrosine previously observed in wheat shoots. It seems likely that higher plants can readily oxidize a number of $\alpha$-hydroxy acids to the corresponding $\alpha$-keto acids which, in turn, are converted to the $\alpha$-amino acids by transamination, and in this connection Kretovich et al. have recently obtained evidence for the formation of aromatic amino acids from corresponding $\alpha$-keto acids by transamination reactions in plants.

Compounds which were good precursors of tryptophan in the present investigation were also good precursors of gramine, but dilution values for gramine formation were larger, as is evident from Table 1. This suggests that the labelled compounds employed were converted via tryptophan. Tryptophan has been shown to be a good precursor of gramine by Marion et al. and although the intermediate steps between tryptophan and gramine are not yet known, Breccia and Marion have reported that 3-indolylacrylic acid is readily converted to gramine in barley seedlings. However, in the present series of experiments this compound was found to be the poorest gramine precursor of all the compounds tested (Table 1). This discrepancy might be due to the different methods used for administration of the labelled compounds. Breccia and Marion fed the precursor through the roots of whole seedlings over long periods of time, while in the present investigation the precursor was fed directly, and comparatively quickly, into the shoots.

The results in Table 1 do not indicate any of the labelled compounds to be possible intermediates between tryptophan and gramine. Any such intermediate would be expected to form gramine with less dilution of C\textsuperscript{14} than shown for conversion of tryptophan to

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gramine. The only compound to do this was serine-3-C14, in one experiment. The relatively high efficiency of this precursor is due to its ability to contribute C14 to all three extranuclear carbons of gramine. It is well known that carbon-3 of serine can enter the "one-carbon" metabolic pool and serve as a source of methyl groups. It also forms the β-carbon atom of tryptophan, which is the carbon of gramine that is attached to the indole nucleus. Radioactive gramine samples were converted to 3-methoxymethylindole and tetramethylammonium iodide by treatment with methyl iodide in methanolic potassium hydroxide at room temperature. The distribution of C14 in gramine formed from serine-3-C14 and from other precursors is shown in Table 2. Serine was the only precursor that gave appreciable labelling of the N-methyl groups of gramine. The labelling pattern of the other gramine samples is as expected if they originated via tryptophan. The overall recovery of C14 in the serine experiment (Table 2) was about 20 per cent low. This is probably due to poor recovery of C14 in the tetramethyl ammonium iodide, since the method gave good recoveries with gramine samples labelled only in the carbon adjacent to the ring (i.e. in the methoxymethylindole).

In conclusion it may be said that aromatic amino acids in plants probably originate by the shikimic acid pathway, as in bacteria. The evidence rests mainly on tracer investigations. Previous investigations in this laboratory have shown that it is likely that phenylalanine and tyrosine are formed by the shikimic acid pathway in plants. The present

### Table 1. Efficiency of C14-labelled Compounds as Precursors of Tryptophan and Gramine

<table>
<thead>
<tr>
<th>Plant group*</th>
<th>Compound fed</th>
<th>Amount fed†</th>
<th>(% of C14 found in ‡)</th>
<th>Dilution of C14 in §</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Shikimic acid-G-C14</td>
<td>8.87 µmoles</td>
<td>0.67</td>
<td>0.29</td>
</tr>
<tr>
<td>A</td>
<td>Anthranilic acid-G-C14</td>
<td>4.96 µmoles</td>
<td>1.76</td>
<td>1.70</td>
</tr>
<tr>
<td>A</td>
<td>l-Serine-3-C14</td>
<td>11.1 µmoles</td>
<td>2.78</td>
<td>0.67</td>
</tr>
<tr>
<td>A</td>
<td>1-Tryptophan-β-C14</td>
<td>15.5 µmoles</td>
<td>0.71</td>
<td>2.81</td>
</tr>
<tr>
<td>B</td>
<td>3-Indolyacetonitrile</td>
<td>12.7 µmoles</td>
<td>0.41</td>
<td>0.16</td>
</tr>
<tr>
<td>B</td>
<td>3-Indolyacrylic acid</td>
<td>8.9 µmoles</td>
<td>2.89</td>
<td>6.67</td>
</tr>
<tr>
<td>C</td>
<td>3-Indolyacetic acid</td>
<td>14.7 µmoles</td>
<td>8.00</td>
<td>0.52</td>
</tr>
<tr>
<td>A</td>
<td>3-Indolyacetic acid</td>
<td>16.7 µmoles</td>
<td>4.80</td>
<td>0.74</td>
</tr>
<tr>
<td>A</td>
<td>3-Indolyacetic acid</td>
<td>24.9 µmoles</td>
<td>0.01</td>
<td>76.2</td>
</tr>
<tr>
<td>A</td>
<td>3-Indolyacetic acid</td>
<td>5.91 µmoles</td>
<td>0.05</td>
<td>0.50</td>
</tr>
<tr>
<td>A</td>
<td>3-Indolyacetic acid</td>
<td>11.5 µmoles</td>
<td>0.05</td>
<td>0.43</td>
</tr>
<tr>
<td>A</td>
<td>3-Indolyacetic acid</td>
<td>10.9 µmoles</td>
<td>4.58</td>
<td>1.21</td>
</tr>
<tr>
<td>B</td>
<td>3-Indolyacetic acid</td>
<td>9.70 µmoles</td>
<td>4.44</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>3-Indolyacetic acid</td>
<td>4.8 µmoles</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>B</td>
<td>3-Indolyacetic acid</td>
<td>11.2 µmoles</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>B</td>
<td>3-Indolyacetic acid</td>
<td>10.8 µmoles</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>B</td>
<td>3-Indolyacetic acid</td>
<td>10.3 µmoles</td>
<td>0.04</td>
<td>0.48</td>
</tr>
<tr>
<td>B</td>
<td>3-Indolyacetic acid</td>
<td>6.52 µmoles</td>
<td>0.02</td>
<td>nil</td>
</tr>
<tr>
<td>C</td>
<td>3-Indolyacetic acid</td>
<td>6.78 µmoles</td>
<td>0.02</td>
<td>nil</td>
</tr>
<tr>
<td>C</td>
<td>3-Indolyacetic acid</td>
<td>7.00 µmoles</td>
<td>0.04</td>
<td>nil</td>
</tr>
</tbody>
</table>

* Plants of group A were grown in December, group B in April and group C in July.
† Per 50 g fresh wt. Tryptophan recovered was 40-65 µmoles; gramine recovered was 80-105 µmoles for group B plants and 130-160 µmoles for groups A and C.
‡ % of C14 fed found in tryptophan or gramine.
§ Specific activity of compound fed (µc per µmole) divided by specific activity of compound isolated.

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results show this is probably true also for tryptophan. However, a considerable amount of work with isolated enzyme systems is now required before more definite conclusions can be drawn.

**TABLE 2. DISTRIBUTION OF C\textsuperscript{14} IN GRAMINE ISOLATED FROM BARLEY FED WITH VARIOUS PRECURSORS**

<table>
<thead>
<tr>
<th>Labelled precursor</th>
<th>% of gramine-C\textsuperscript{14} found in 3-methoxymethyl indole</th>
<th>% of gramine-C\textsuperscript{14} found in tetramethylammonium iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthranilic acid-G-C\textsuperscript{14}</td>
<td>96</td>
<td>—</td>
</tr>
<tr>
<td>Indole-2-C\textsuperscript{14}</td>
<td>95</td>
<td>—</td>
</tr>
<tr>
<td>L-Serine-3-C\textsuperscript{14}</td>
<td>28</td>
<td>49</td>
</tr>
<tr>
<td>DL-Tryptophan-(\beta)-C\textsuperscript{14}</td>
<td>101</td>
<td>0.3</td>
</tr>
<tr>
<td>Gramine-C\textsuperscript{14}</td>
<td>95</td>
<td>—</td>
</tr>
<tr>
<td>3-Indolylactic acid</td>
<td>95</td>
<td>—</td>
</tr>
<tr>
<td>DL-3-Indolyllactic acid</td>
<td>103</td>
<td>—</td>
</tr>
</tbody>
</table>

**EXPERIMENTAL**

**Preparation of C\textsuperscript{14}-labelled Compounds**

Sodium formate-C\textsuperscript{14}, formaldehyde-C\textsuperscript{14}, L-serine-3-C\textsuperscript{14}, DL-tryptophan-\(\beta\)-C\textsuperscript{14} and generally labelled samples of L-phenylalanine and toluene were purchased from Atomic Energy of Canada Ltd. Shikimic acid (generally labelled),\textsuperscript{18} DL-phenylalanine-\(\beta\)-C\textsuperscript{14} and L-phenylalanine-\(\beta\)-C\textsuperscript{14} were prepared as described previously.\textsuperscript{20}

Indole-2-C\textsuperscript{14} was synthesized from sodium formate-C\textsuperscript{14} as described by Leete and Marion.\textsuperscript{18} Gramine, labelled in the carbon attached to the ring, was synthesized from formaldehyde-C\textsuperscript{14} by the procedure of Kühn and Stein.\textsuperscript{21} Portions of this gramine were used for the preparation of DL-3-indolylactic acid by the procedure of Gortatowski and Armstrong,\textsuperscript{22} for the preparation of 3-indolealdehyde by Thesing's procedure,\textsuperscript{23} and for the preparation of 3-indolylacetonitrile by the procedure of Thesing and Schulde.\textsuperscript{24} The indolylacetonitrile was used for preparation of tryptamine by lithium aluminum hydride reduction\textsuperscript{25} and for preparation of 3-indolylacetic acid by alkaline hydrolysis followed by acid hydrolysis.\textsuperscript{26} Hypochlorite oxidation of DL-tryptophan-\(\beta\)-C\textsuperscript{14} gave labelled 3-indolylacetaldheyde.\textsuperscript{27} These procedures all give the 3-indolyl derivatives labelled in the carbon attached to the ring. The procedures were scaled down to 0.5 to 1.0 m mole. The yields were approximately as reported for the larger scale described in the literature. The purity was tested by chromatography on paper with an appropriate solvent. Radioautographs of these chromatograms showed one radioactive spot with the correct \(R_f\) in each case.

A sample of 3-indolylacrylic acid was prepared from 3-indolealdehyde (100 mg) by the procedure of Bauguess and Berg.\textsuperscript{28} The crude acid (96 mg, m.p. 172–175\textdegree) was purified by

chromatography on a 2.5 × 21 cm column of cellulose powder using isopropanol: conc. ammonium hydroxide: water (20 : 1 : 3) as the eluting agent. Fractions containing the indolylacrylic acid were pooled, evaporated under reduced pressure, the solid residue dissolved in a small volume of 0.2 N sodium hydroxide and the purified product precipitated by hydrochloric acid. This gave 71 mg (55 per cent); m.p. 175-178°. (Found: C, 70.47; H, 4.89. C₁₁H₂₈NO₄ requires: C, 70.58; H, 4.85 per cent). The melting point is about 20° lower than reported previously but it agrees with that of a sample of 3-indolylacrylic acid purchased from the Sigma Chemical Company. This melting point could not be raised by recrystallization. A sample (m.p. 175-178°) was reduced in ethanol by hydrogen in a simple glass apparatus using palladium (5 per cent)-charcoal as catalyst. The hydrogen uptake was 97 per cent of theory and 3-indolylpropionic acid, m.p. 133-134° was isolated in good yield. This agrees with the m.p. of 3-indolylpropionic acid obtained by Bauguess and Berg. Paper chromatography of the C₁⁴-labelled indolylacrylic acid, followed by radioautography, showed only one radioactive spot with the same mobility as unlabelled indolylacrylic acid.

Anthranilic acid-G-C₁⁴ was made from toluene-G-C₁⁴ by a procedure involving mononitration of the toluene, oxidation of the mixed ortho and para nitrotoluenes to the corresponding benzoic acids, reduction of the mixed nitrobenzoic acids to the amino-benzoic acids and chromatographic separation of the amino-benzoic acids. The following procedure gave the best yields of several modifications tried.

Generally labelled toluene (167 mg, 300 µc) in a 1.8 × 11 cm glass-stoppered tube, was treated with a well-cooled mixture of sulphuric acid (259 mg) and nitric acid (s.g. 1.42, 173 mg). The tube was stoppered and the reaction mixture stirred with a Teflon-covered magnetic stirrer bar, for 18 hr at room temperature. The reaction mixture was diluted with 20 ml of water and extracted with 3 × 20 ml portions of ethyl ether. The combined ether extract was washed with 25 ml of 0.1 M sodium carbonate, then with water, dried over sodium sulfate, filtered and the ether evaporated, to yield a mixture of ortho and para nitrotoluene (224 mg). To this mixture in a 50 ml round-bottomed flask, fitted with a reflux condenser, was added 1.6 ml of 5 N sodium hydroxide, 20 ml of water and 210 mg of potassium permanganate. The mixture was refluxed until the pink color disappeared (20 min); three further additions of potassium permanganate (210 mg) were made. After the last addition of potassium permanganate, the color remained after 30 min of refluxing so the oxidation was considered to be finished. The reflux condenser was changed to distillation and 10 ml of distillate, containing the unoxidized nitrotoluene, was collected. The hot residue in the round-bottom flask was filtered and the cake was washed with 2 × 15 ml portions of boiling water. The filtrate was cooled, acidified with concentrated hydrochloric acid and extracted with 3 × 30 ml portions of ethyl ether. The combined ether extract was washed with water, dried over sodium sulfate, filtered and the ether evaporated. The remaining residue (ortho and para nitrobenzoic acids) weighed 160 mg and had a m.p. 132-155°.

The mixture of ortho and para nitrobenzoic acids was dissolved in 25 ml of ethanol and placed in a low pressure hydrogenation apparatus, 15 mg of Adams platinum oxide catalyst was added, and the mixture was hydrogenated at 15 lb pressure. The reaction stopped in 6 min. The catalyst was removed by filtration and the filtrate evaporated to dryness. The resulting residue, a mixture of p-aminobenzoic acid and o-aminobenzoic

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acid (anthranilic acid), weighed 129 mg. The aminobenzoic acid isomers were separated on 2.5 x 25 cm cellulose column by elution with n-butanol saturated with 1.5 N ammonium hydroxide. The anthranilic acid came off the column first and was widely separated from the p-aminobenzoic acid. The fractions containing the anthranilic acid were combined, the solvent was removed by evaporation, and the residue sublimed at 95° and 2 mm pressure. The yield of anthranilic acid was 51 mg (20 per cent) m.p. 142-143°. The p-aminobenzoic acid was also recovered and crystallized from water. Paper chromatography showed the anthranilic acid to be pure.

Cultivation of Plants

Young plants of barley (*Hordeum vulgare, L. var. Atlas 1-35-3) were grown in a greenhouse with controlled temperature. The subirrigation gravel culture method was used with a modified Hoagland solution. Additional illumination was supplied by an overhead bank of “cool white” fluorescent tubes to give a daylength of 18 hr and a minimum light intensity of 7000 lux. The temperature was 18–20° during the day and 14–16° at night. The plants were used when 10 days old.

Administration of C14-labelled Compounds

The labelled compound was dissolved in about 20–30 ml of water, the pH adjusted to 6.5–7.0 and the solution divided equally between two 250 ml beakers. The shoots of the seedlings were separated from the roots by cutting under water with a razor blade. These shoots (50 g fresh weight) were distributed equally between the two beakers with the cut ends submerged in the solution of labelled compound. The beakers were put in a growth chamber illuminated by light (12,000 lux) from “cool white” fluorescent tubes. The solutions were nearly all absorbed in 6 hr. Distilled water (10 ml) was then added to each beaker and after it was nearly all absorbed another 10 ml was added. When this “washing-in” procedure was completed an excess of distilled water was added and the shoots allowed to metabolize. The total time allowed, including that for absorption, was 72 hr. During this period a daylength of 18 hr was used and the temperature kept at 23° in the day and 18° at night.

Extraction of Plant Material and Recovery of Gramine

At the end of the metabolic period (72 hr) the plant material (50 g) was extracted with cold methanol (600 ml) in a Waring blender. The homogenate was allowed to stand 24 hr at -5° and was then filtered on a Buchner funnel. The fibrous residue was washed with cold methanol, followed by absolute ethanol, air-dried and saved for recovery of bound tryptophan.

The filtrate was evaporated at 40° and 15 mm pressure, to a volume of about 200 ml, extracted three times with 200 ml portions of petroleum ether (30–60°) and the petrol extracts discarded. The remaining aqueous solution was evaporated to dryness under an air stream at room temperature. The dry residue thus obtained was taken up in 40 ml of N sulphuric acid and extracted four times with 60 ml portions of diethyl ether and the ether extracts discarded. The remaining aqueous solution was cooled in ice and 10 ml of 10 M sodium hydroxide was added. The resulting alkaline mixture was re-extracted

*L. Jacobson, Plant Physiol. 26, 411 (1951).*
with four 100 ml portions of diethyl ether. These ether extracts contained the gramine. They were pooled, washed with water, dried over anhydrous sodium sulphate, filtered and the ether evaporated. The residue was rinsed into a sublimation apparatus and the gramine sublimed at 90° and 1·0 mm pressure. The product had a m.p. 122–128°. After recrystallization from benzene the m.p. was 133° and was not depressed when the compound was mixed with an authentic sample of gramine.

Hydrolysis of Protein and Recovery of Tryptophan

The fibrous residue from the above extraction procedure (about 2 g) was mixed with 5 N sodium hydroxide (30 ml) in a 25 x 100 mm test tube. The tube was sealed and the contents digested for 24 hr at 100°. After cooling to room temperature the tube was opened and the contents filtered through Analytical Celite filter aid, the cake being washed four times with 30 ml of cold water. The filtrate was evaporated under an air stream to about 30 ml. The pH was adjusted to 6·0 with 10 N sulphuric acid. On addition of 50 ml of ethanol a precipitate formed which was removed by filtration after standing 2 hr at room temperature. The filter cake was washed with 80 per cent ethanol, then with absolute ethanol and discarded.

The filtrate was evaporated to dryness, taken up in 20 ml of water and passed through a 1·2 x 16 cm column of Amberlite 1R-120-H ion exchange resin. The column was washed with water and the washings discarded. The amino acids were eluted from the column with 100 ml of 1·5 M ammonium hydroxide and the eluate evaporated to dryness. The residue was taken up in 0·5 N acetic acid (3 ml) and passed through a 2 x 60 cm column of Dowex-l-acetate using 0·5 N acetic acid as the eluting agent. The tryptophan fractions were combined and checked for purity by paper chromatography with two solvents: n-butanol : acetic acid : water (4 : 1 : 1·8) and isopropanol : conc. ammonium hydroxide : water (20 : 1 : 3). Only one ninhydrin-positive spot could be detected, and it had the same mobility as tryptophan. The tryptophan was measured quantitatively by the method of Moore and Stein.s The remainder of the sample (10 mg) was evaporated to dryness and dissolved in 2 ml of hot water, half the water was evaporated under an air stream at room temperature and 0·2 ml of ethanol was added. After standing 18–20 hr at 3° the crystals were filtered off, washed with 10 per cent ethanol, with absolute ethanol and finally with ether, and then dried to constant weight (6 mg) in a vacuum desiccator. Three parts of carrier (DL-tryptophan) was added and the whole dissolved in hot water, evaporated by an air stream at room temperature and dried to constant weight in a desiccator.

Degradation of Gramine

Radioactive gramine samples were converted to 3-methoxymethylindole and tetramethylammonium iodide by treatment with methyl iodide in methanolic potassium hydroxide at room temperature. Both products were recovered and analysed for C14. This procedure was tested on gramine synthesized as described above. The methoxymethylindole contained 99·5 per cent of the C14, whereas the tetramethylammonium iodide was inactive. This procedure seems to be reliable for measuring the C14 in the indole ring and the attached carbon, but it was not tested on synthetic gramine labelled in the N-methyl groups, since such a sample was not available.

Measurement of C\textsuperscript{14}

The samples were converted to carbon dioxide by combustion with the Van Slyke reagent\textsuperscript{[8]} and the activity of the resulting carbon dioxide was measured by a dynamic condenser electrometer (a Nuclear-Chicago Dynacon).

Acknowledgement—The authors are grateful to Mr. J. Dyck for numerous C\textsuperscript{14} analyses.