Isolation and Characterization of Tryptophan Transaminase and Indolepyruvate C-Methyltransferase

ENZYMES INVOLVED IN INDOLMYCIN BIOSYNTHESIS IN STREPTOMYCES GRISEUS*

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Two enzymes, tryptophan transaminase and indolepyruvate C-methyltransferase, which are active in the initial steps of the biosynthetic pathway of the antibiotic indolmycin, have been detected and partially purified from cell-free extracts of Streptomyces griseus. The transaminase has been purified 8-fold by ammonium sulfate fractionation. At this stage of purification, it catalyzes the α-ketoglutarate and pyridoxal phosphate-dependent transamination of L-tryptophan, 3-methyltryptophan, L-phenylalanine, and L-tyrosine.

The C-methyltransferase catalyzes the transfer of a methyl group from S-adenosylmethionine to position 3 of the aliphatic side chain of indolepyruvate. No cofactors are required. The C-methyltransferase has been purified 110-fold by ammonium sulfate fractionation, Sephadex G-150 gel filtration, DEAE-Sephadex column chromatography, and Bio-Gel A-5m gel filtration. The enzyme has a broad pH optimum of 7.5 to 8.5. A molecular weight of 55,000 ± 5,000 has been determined by Sephadex G-200 gel filtration with reference proteins and a molecular weight of 58,500 ± 8,000 has been determined by sucrose density gradient centrifugation. The enzyme is relatively stable at temperatures of 0-5°C but is destroyed by freezing or by heating. The C-methyltransferase is inhibited strongly by the thiol reagents p-chloromercuribenzoate and N-ethylmaleimide. The Zn²⁺ and Fe²⁺ chelators 1,10-phenanthroline and 2,2'-bipyridine also inhibit the enzyme activity but EDTA does not. Michaelis-Menten constants have been determined for the 110-fold purified enzyme as 1.2 × 10⁻⁴ M for S-adenosylmethionine and 4.8 × 10⁻⁴ M for indolepyruvate. The enzyme activity in the crude extract is inhibited competitively by indolmycin (Kᵢ = 2.3 mM) and L-tryptophan (Kᵢ = 0.17 mM), but these effects are not observed after the enzyme has been passed through the Sephadex G-150 column during purification. The crude extract is capable of methylating phenylpyruvate and p-hydroxyphenylpyruvate but this capability is lost upon purification of the indolepyruvate C-methyltransferase activity. No methylation of L-tryptophan occurs under the conditions used.

The antibiotic indolmycin (Fig. 1) is produced by Streptomyces griseus (ATCC 12648). It exhibits antimicrobial activity against gram-positive and gram-negative bacteria, including polyresistant staphylococci (1-3). The culture characteristics of the indolmycin-producing strain of S. griseus have been described (2), and a fermentation procedure for the production of indolmycin together with two other antibiotics of unknown structure has been patented (4). The structure (5), relative (3, 5) and absolute (6, 7) stereochemistry of indolmycin have been determined and syntheses of indolmycin and some of its degradation products and analogs have been reported (3, 5).

Previous studies have established that indolmycin is derived biosynthetically from tryptophan, the methyl group of methionine, and the amidino group of arginine (7). A hypothetical pathway for the biosynthesis of indolmycin, as outlined in Fig. 2, and experiments supporting the postulated pathway have been reported by Hornemann et al. (7). The postulated biosynthesis involves transamination of tryptophan to yield...
indolepyruvic acid, followed by the transfer of an intact methyl group from methionine to position 3 of the indolepyruvic acid side chain to yield 3-methylindolepyruvate. This intermediate is reduced to give indolmycenc acid. The steps from indolmycenc acid to indolmycin have not yet been fully elucidated. Presumably, however, the amidino group of arginine condenses is reduced to give indolmycenc acid. The steps from indolmycin to indolmycin have not yet been fully elucidated.

This paper describes the partial purification and characterization of two enzymes, a tryptophan transaminase (tryptophan:α-ketoglutarate aminotransferase) and an indolepyruvate C-methyltransferase (S-adenosylmethionine:indolepyruvate C-methyltransferase), from cell-free extracts of indolmycin-producing S. griseus. These enzymes apparently are involved in the initial steps of the biosynthesis of indolmycin.

The C-methyltransferase was especially of interest for several reasons. First, it is a likely candidate for being a regulatory enzyme in the biosynthesis of indolmycin since it is probably the first enzyme to commit substrate irreversibly to the pathway leading to this antibiotic. Second, biological C-methylation reactions are of general interest since they participate in the biosynthesis of many compounds of biochemical importance, e.g. nucleic acids, vitamin K, and ergosterol, and because they pose some interesting mechanistic questions (8).

A few C-methyltransferases have been reported previously including rat spleen DNA methylase which catalyzes the formation of 5-methylcytosine residues (9), demethylmenaquinone methylase (10), an enzyme involved in flavin biosynthesis (11), and a Δ^4-sterol methyltransferase (12), but little is known about the mechanism of these reactions except that they generally require no cofactors and are irreversible. Furthermore, there are a number of antibiotics which contain methyl-branched amino acids which conceivably could arise from C-methylation of amino acid derivatives in a manner analogous to the formation of 3-methylindolepyruvate in the indolmycin pathway. Such amino acids and related moieties found to occur in nature include indolepropionic acid (13), 3-methyltryptophan in the peptide antibiotic telomycin (14), 3-methylphenylalanine, 3-methylvaline, and 3-methylproline, components of the peptide antibiotic bottromycin A (15); and N^6,β-dimethylleucine which is a component of the peptide antibiotics etamycin (16) and triostin C (17). In two of these branches the methyl group has been shown to originate from the methyl group of methionine (13, 18, 19).

**EXPERIMENTAL PROCEDURE**

**Methods**

**Growth of Cultures and Preparation of Crude Cell-free Extract**

The strain Streptomyces griseus (Krainsky) Waksman and Henrici sensu Pridham (20) (ATCC 12648) was used as the source of the enzymes. The organism was maintained on slants of Emerson agar at 24°C. For production of cells to be used in enzyme preparations, the culture was first grown on a medium described by Rao (21) which contained dextrose, 1.0 g; K₂HPO₄, 0.5 g; NaCl, 0.2 g; CaCO₃, 0.2 g; distiller's solubles (Seagrams and Co.), 0.20 g; soybean meal (Lafayette Cooperative Elevator Co.), 0.15 g; and distilled water to 100 ml. A part of the mycelial pad from a slant culture was transferred under sterile conditions to a 500-ml Erlenmeyer flask containing 100 ml of the above medium and was allowed to grow for 4 to 7 days on a rotary shaker at 180 rpm at 24°C. From this culture 2-ml aliquots were withdrawn and transferred to 500-ml Erlenmeyer flasks containing “Phytone” (Baltimore Biological Laboratories), 2.0 g; yeast extract (Difco), 0.2 g; trace element solution, 0.1 ml; FeCl₃, 0.1 mg; and distilled water to 100 ml. The trace element solution had the following composition: AlK(SO₄)₃, 0.015%; KI, 0.003%; KBr, 0.003%; MnCl₂·4H₂O, 0.040%; ZnSO₄, 0.005%; CaSO₄, 0.000%; CoCl₂, 0.007%; (NH₄)₆MoO₄·4H₂O, 0.003%; K₂CrO₄, 0.001%; CuSO₄·5H₂O, 0.003%. After 30 to 42 hours of growth in the latter medium, the mycelium was harvested by vacuum filtration and washed twice with distilled water.

The mycelium (1.5 g wet weight per flask) was suspended in 10 ml/g of 0.01 M phosphate buffer, pH 7.0. The cells were broken by one passage through a French pressure cell at 15,000 to 20,000 p.s.i., or by sonification with a Branson Sonifier (model W 185) at Setting 3 for 5 min at 5 - 10°C. The resulting suspension was centrifuged at 30,000 × g for 20 min to remove cell debris and the supernatant was dialyzed for 1 hour at 4°C against each of three changes of 1 liter of the same buffer. The protein concentration of the resulting cell-free extracts was 7 to 10 mg/ml as determined by the biuret method (22).

**Enzyme Assays**

The transaminase activity was assayed by two methods, one measuring conversion of radioactive tryptophan into radioactive indolepyruvate, and the other measuring the formation of indolepyruvate by its absorbance at 305 nm.

For the radioactive assay, 1 μmol of L-[14C]tryptophan, 1 μmol of α-ketoglutarate, 0.03 μmol of pyridoxal phosphate, and 1 ml of enzyme extract in 0.01 M phosphate buffer, pH 7.0. Total volume 1.38 ml were incubated at 30°C for 1 hour. All substrate solutions were adjusted to pH 7.0 before addition to the reaction mixture. After acidification of the reaction mixture, radioactive indolepyruvate was extracted with ethyl acetate and an aliquot of the ethyl acetate phase was counted for radioactivity.

A spectrophotometric assay was developed based on the absorbance of the enol form of indolepyruvate in neutral solution at 305 nm (see Fig. 3). The usual reaction mixture consisted of 1 ml of buffered enzyme extract, pH 7.0, 19 μmol of α-ketoglutarate, 0.03 μmol of pyridoxal phosphate, and 10 μmol of L-tryptophan, added at the start of the reaction, in a total volume of 1.4 ml. The increase in absorbance at 305 nm was measured in a Zeiss Spectrophotometer. The molar extinction coefficient of indolepyruvate is 17,000 M⁻¹ cm⁻¹ under these conditions. Therefore an increase in absorbance of 0.17 absorption unit represents the formation of 1 nmol/ml of product. No correction for the contribution of L-tryptophan to the absorbance at 305 nm was made in this assay.

C-Methyltransferase activity was determined by incubation of 0.05 μCi of S-adenosyl-L-[methyl-¹⁴C]methionine or S-adenosyl-L-[methyl-¹³C]methionine together with 0.25 to 1.0 μmol of unlabeled S-adenosyl-L-methionine and 0.25 to 1.0 μmol of indolepyruvic acid (brought to the pH of the reaction mixture with 0.2 N NaOH before addition) and 1.0

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Fig. 1. Indolmycin.

Fig. 2. Hypothetical pathway for indolmycin formation.

[Diagram of indolmycin formation with relevant chemical structures.]
Bio-Gel stage, varying concentrations of one substrate and 0.01 M the substrates: 25 μl of enzyme (1 μg of protein/μl) purified through the following manner to determine the Michaelis-Menten constants of the counts obtained in the control incubation were subtracted from boiled enzyme was run with each set of reactions and where necessary the later stages of purification were used. A control incubation using grams was detected using a Packard model 7201 radiochromatogram counted to at least 2% statistical error. Radioactivity on chromato-

was removed by repeated addition of unlabeled methanol and evapora-

LS-100 and Beckman LS-250 liquid scintillation spectrometers. Sam-

indolepyrazolyl]

was determined by the biuret method (22) or during the later stages of purification is summarized in Table I.

Properties of Tryptophan Transaminase

Materials

Indolmycin was a gift from Chas. Pfizer and Co. Isomers of 3-methyltryptophan were provided by Dr. H. R. Snyder, Urbana, Ill. Indolmycinic acid was synthesized by Dr. Laurence Hurley following the procedure of Preobrazhenskaya et al. (3). Indolepyruvic acid was obtained from Aldrich Chemical Co. Other nonradioactive chemicals and biochemicals were obtained from commercial sources, primarily Sigma and Calbiochem, and were of reagent grade. S-Adenosyl-L-

RESULTS

3-Methyltryptophan, besides functioning as a substrate, was also observed to inhibit the tryptophan transaminase activity. This could be measured using the spectral assay since neither

centrifugation at 30,000 × g, ammonium sulfate was added to the supernatant in a similar manner to achieve 60% saturation, keeping the pH at 7.0. The protein that precipitated between 40 and 60% saturation contained 77% of the initial units of transaminase activity and 26% of the initial protein, and represented a 2.9-fold purification of the transaminase activity. The protein was dissolved in 5 to 10 ml of 0.01 M phosphate buffer, pH 7.0, and dialyzed against three changes of 1 liter of the same buffer for 1 hour each. The transaminase was not purified further.

C-Methyltransferase

Step 1. Ammonium Sulfate Treatment The initial step in the purification of the C-methyltransferase was an ammonium sulfate precipitation performed in a manner similar to that described above for the transaminase, except that 0.1 mM Cieiald’s reagent was added to the crude enzyme extract and the protein precipitating between 35 and 55% saturation was collected. This precipitate was dissolved in 5 to 10 ml of 0.01 M phosphate buffer, pH 7.0, and dialyzed for 3 hours against two changes of 1 liter of the same buffer.

Step 2. Sephadex Chromatography—The dialyzed solution containing the C-methyltransferase was applied to a column (2.5 × 33 cm) of Sephadex G-150 which had been equilibrated with 0.01 M phosphate buffer, pH 7.0, and was eluted with the same buffer. Fractions of 5 ml each were collected. The C-methyltransferase was recovered in Fractions 15 through 22. The total volume of the active fractions was reduced to approximately 4 ml using an Amicon pressure dialysis apparatus.

Step 3. DEAE-Sephadex Chromatography—The combined fractions from the previous step were applied to a column (1.8 × 23 cm) of DEAE-Sephadex which had been equilibrated with 0.05 M phosphate buffer, pH 6.8. A 500-ml gradient of 0 to 0.4 M NaCl in 0.05 M phosphate buffer, pH 6.8, was used to elute the column. Fractions of 5 ml each were collected and monitored for protein and C-methyl-

transferease activity. The volume of the combined active fractions was reduced to approximately 5 ml using an Amicon pressure dialysis apparatus.

Step 4. Bio-Gel A-5m Chromatography—The enzyme preparation from the preceding step was applied to a column (1.0 × 25 cm) of Bio-Gel A-5m which had been equilibrated with 0.01 M phosphate buffer, pH 7.0, and subsequently was eluted with the same buffer. One-milliliter fractions were collected. The C-methyltransferase activity was recovered in Fractions 9 through 12, coincident with a well defined protein peak.

By the above procedure, the C-methyltransferase may be purified approximately 110-fold with an over-all yield of 40 to 45%. A typical purification is summarized in Table I.

Enzyme Purification Procedures

All steps were carried out at 0-4° unless otherwise specified.

Transaminase—For purification of the transaminase solid ammonium sulfate (enzyme grade) was added to the supernatant in the presence of 0.03 M pyridoxal phosphate over a period of 15 min to give 45% saturation. The pH was adjusted to 7.0 with solid NaOH, and the solution was stirred for a further 40 min. Following 20 min of centrifugation at 30,000 × g, ammonium sulfate was added to the supernatant in a similar manner to achieve 60% saturation, keeping the pH at 7.0. The protein that precipitated between 40 and 60% saturation contained 77% of the initial units of transaminase activity and 26% of the initial protein, and represented a 2.9-fold purification of the transaminase activity. The protein was dissolved in 5 to 10 ml of 0.01 M phosphate buffer, pH 7.0, and dialyzed against three changes of 1 liter of the same buffer for 1 hour each. The transaminase was not purified further.

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3-methyltryptophan nor its transaminated product, 3-methylindolepyruvate, absorb at 305 nm. (2RS,3SR)-3-Methyltryptophan present at a concentration of 10 mM (L-tryptophan was also present in 10 mM concentration) inhibited the rate of transamination of L-tryptophan by 48% while (2RS,3RS)-3-methyltryptophan under identical conditions caused 38% inhibition. Addition of both isomers of 3-methyltryptophan resulted in 64% inhibition of L-tryptophan transamination activity. These results are compatible with those obtained with the radioactive assay and suggest that L-tryptophan and 3-methyltryptophan are transaminated by the same enzyme. Apparently the enzyme is stereospecific only for position 2 of 3-methyltryptophan.

Identification of the unstable product of the reaction, indolepyruvate, was achieved upon reduction with sodium borohydride to indolelactic acid and thin layer chromatographic identification as the methyl ester in ligroin/l-octanol/acetone (8/2/1). When radioactive tryptophan was used in the reaction mixture in concentrations of 5 x 10^{-3} M and 1 x 10^{-3} M ammonium sulfate precipitate in a phosphate buffer solution. cell-free extract with 10% glycerol or by freezing the 45 to 60% graphic identification as the methyl ester in ligroin/l-octanol/acetone.

The enzyme is best stored at -20° by freezing the crude extract that co-chromatographs and co-crystallizes with authentic S-adenosylmethionine with sodium borohydride yielded a compound that co-chromatographs and co-crystallizes with authentic indolmycinic acid. Kuhn Roth oxidation yielded acetic acid with a specific radioactivity identical with that of the indolmycinic acid.

3. Mild oxidation with dilute hydrogen peroxide yielded indoleisopropionic acid which could be co-crystallized with authentic carrier (R)-indoleisopropionic acid as the cinchonine salt.

The methods and results of the identifications are discussed in detail in a previous publication (24). The identification procedures were carried out initially using crude enzyme and Procedures 1 and 2 as indicated above were repeated using purified C-methyltransferase.

Purification steps carried out as described and summarized in Table I resulted in a 110-fold purification of the enzyme activity. Characterization of the properties of the enzyme as described in the following section was performed with enzyme that stage of purification unless otherwise specified.

**Specificity**—When phenylpyruvate or p-hydroxyphenylpyruvate was substituted for indolepyruvate in the reaction mixture with crude enzyme preparation, the reaction proceeded at rates of 49 and 35%, respectively, of the reaction rate with indolepyruvate. No reaction occurred when L-tryptophan exceeded at rates of 49 and 35%, respectively, of the reaction rate with indolepyruvate. No reaction occurred when L-tryptophan was substituted. However, ammonium sulfate precipitation of the indolepyruvate C-methyltransferase activity resulted in a 45% loss of initial p-hydroxyphenylpyruvate and phenylpyruvate C-methyltransferase activity and after purification on Sephadex G-150, only 14% of the initial activity remained. Curiously, no phenylpyruvate C-methyltransferase activity could be detected in any other ammonium sulfate fraction. This would indicate that either the enzyme is nonspecific in its natural form and undergoes a conformational or other change during purification, losing activity toward phenylpyruvate, or alternatively, that two enzymes are responsible for the dual activity and the phenylpyruvate-specific enzyme is denatured during the purification procedure.

**pH Dependence**—The purified enzyme shows optimal activity between pH 7.5 and 8.5. There appears to be some dependency upon the buffer used since in phosphate buffer the enzyme is somewhat more active at pH 7.5 than at pH 8.0, while in other buffers, the optimum pH is slightly higher.

The enzyme is inactivated irreversibly at pH values of 5.5 and below. The longer it remains at pH 5.5 the less able it is to recover activity upon neutralization to pH 7.0. For example, neutralization of an enzyme solution that has been kept at pH 5.5 for 1 hour results in a 46% loss of activity compared to a control that has been kept at pH 7.0 continuously. Storage for 2 days at pH 5.5 results in only 6% restoration of activity upon neutralization to pH 7.0.

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**Table I**

**Summary of purification procedure for**

**S-adenosylmethionine:indolepyruvate 3-methyltransferase**

| Fraction | Total protein | Activity | Specific activity | Yield |
|----------|--------------|----------|------------------|-------|
|          | mg           | millionls| millionls/mg (%) |       |
| 1. Crude extract | 358.0 | 28.0 | 0.078 | 100 |
| 2. Ammonium sulfate | 171.0 | 22.5 | 0.13 | 81 |
| 3. Sephadex G-150 | 40.2 | 22.0 | 0.55 | 78 |
| 4. DEAE-Sephadex | 2.2 | 12.8 | 5.82 | 46 |
| 5. Bio-Gel A-5m | 1.5 | 12.5 | 8.30 | 45 |

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**Table II**

**Specificity of transaminase**

| Substrates* | Conversion |
|-------------|------------|
| L-Tryptophan + α-ketoglutarate | 4.24 |
| L-Tyrosine + α-ketoglutarate | 3.50 |
| L-Phenylalanine + α-ketoglutarate | 3.44 |
| L-Tryptophan + α-ketoglutarate | 0 |
| (2RS,3SR)-3-Methyltryptophan + α-ketoglutarate | 2.62 |
| (2RS,3RS)-3-Methyltryptophan + α-ketoglutarate | 1.58 |
| L-Tryptophan + oxaloacetate | 0 |
| L-Tryptophan + pyruvate | 0.3 |

* All substrates were present in 1 mM concentration.
* These values would be doubled if one considers that the enzyme probably can only react with the 2 S form.
Molecular Weight—Two methods were used to determine the molecular weight of the enzyme. Using gel filtration on a calibrated Sephadex G-200 column (1.8 × 30 cm), C-methyltransferase activity eluted at a volume corresponding to a molecular weight of 55,000 ± 5,000, assuming that the enzyme is a globular protein.

The molecular weight was also determined by ultracentrifugation in a 5 to 20% sucrose density gradient calibrated with yeast alcohol dehydrogenase (M₀ 150,000, s₂₀,w = 7.4) and lysozyme (M₀ 17,200, s₂₀,w = 2.1) according to the procedure described by Martin and Ames (25). Using the formula $s/\sqrt{s} = (M₀/M₉)₁/₂$ a molecular weight of 58,500 ± 8,000 was estimated for the C-methyltransferase.

Stability—Both the crude enzyme preparation and the more purified preparations were found to be stable at 2° for periods up to 1 month. Freezing the buffered enzyme preparations caused a partial loss of activity. The enzyme activity was not affected when the ammonium sulfate-precipitated pellet was frozen for a short period of time but after 1 year of storage the reconstituted protein had only 20% of its original activity.

Heat was found to destroy enzyme activity. Heating the crude enzyme preparation to 55° for 5 min resulted in an 85% loss of activity.

Kinetic Experiments—The enzyme was found to follow Michaelis-Menten kinetics. The double reciprocal plots gave Kₘ values of 1.2 × 10⁻⁶ M for S-adenosylmethionine and 4.8 × 10⁻⁶ M for indolepyruvate. The Kₘ value for indolepyruvate was also determined using a crude enzyme preparation and was found to be very close (4.0 × 10⁻⁶ M) to the value determined with the purified enzyme.

Kinetic Experiments—Metal chelators were added to the reaction mixture prior to the addition of substrates. The results are summarized in Table III. As indicated in this table, o-phenanthroline and 2,2'-bipyridine in concentrations from 0.5 to 0.2 mM partially inhibited the enzyme activity. o-Phenanthroline and 2,2'-bipyridine are known to chelate Zn²⁺ and Fe³⁺ and have been shown to inhibit enzymes requiring these metals (26). Diethyldithiocarbamate, which also chelates Zn²⁺ and Fe³⁺ but more specifically Cu²⁺, does not inhibit C-methyltransferase activity at levels up to 2.0 mM and actually appears to slightly stimulate activity. The reason for the apparent inhibition by o-phenanthroline and 2,2'-bipyridine, but not by diethyldithiocarbamate, is not known. However, similar selectivity of inhibition has been observed in other enzyme systems such as a zinc-containing DNA-dependent RNA polymerase from Escherichia coli (27).

TABLE III

| Compound added         | Concentration | Relative activity |
|------------------------|---------------|-------------------|
| 1.10-Phenanthroline    |               |                   |
| 1.0 mM                 | 60            |                   |
| 2.0 mM                 | 40            |                   |
| 0.5 mM                 | 81            |                   |
| 2.2'-Bipyridine        |               |                   |
| 0.5 mM                 | 91            |                   |
| 1.0 mM                 | 72            |                   |
| 2.0 mM                 | 69            |                   |
| 5.0 mM                 | 65            |                   |
| Diethyldithiocarbamate |               |                   |
| 0.5 mM                 | 94            |                   |
| 1.0 mM                 | 112           |                   |
| 2.0 mM                 | 110           |                   |
| Ethylenediaminotetraacetic acid | 101  |                   |
| 0.5 mM                 | 100           |                   |
| 2.0 mM                 | 102           |                   |

Inhibition Studies—Metal chelators were added to the reaction mixture in concentrations as high as 5.0 mM did not inhibit the C-methyltransferase activity. Therefore divalent cations such as Mg²⁺, Mn²⁺, and Ca²⁺ are not essential for the activity of the enzyme.

The thiol group reagent p-chloromercuribenzoate strongly inhibits enzyme activity at 10 μM concentration (see Table IV). This inhibition is reversed by the addition of 1.0 mM cysteine. N-Ethylmaleimide, present at a concentration of 4 mM, causes 52% inhibition of C-methyltransferase activity, thus further supporting the conclusion that a —SH group is important to the enzyme action. However, the thiol group alkylating agents iodoacetate and iodoacetamide inhibit enzyme activity only slightly as indicated in Table IV. According to Dixon and Webb (29) these reagents are not as reactive nor as specific as p-chloromercuribenzoate. Their failure to inhibit the C-methyltransferase therefore does not rule out the involvement of a —SH group in the enzyme's action.

As seen in Table V, the indolepyruvate C-methyltransferase activity in the crude enzyme preparation is inhibited by several compounds including L-tryptophan, L-tryptophanylglycine, L-tryptophan amidic, and indolmycin, and is activated by dimethylsulfoxide and possibly by N-acetyl-L-tryptophan. Dixon (28) plots of the inhibition at various concentrations of indolepyruvate indicate that indolmycin and L-tryptophan are competitive inhibitors for indolepyruvate with Kᵢ values of 2.3 and 0.17 mM, respectively. It was found, however, that the C-methyltransferase is no longer inhibited by any of the above-mentioned effectors after purification through the Sephadex G-150 column step. The loss of inhibition does not appear to be due to aging since aging the enzyme in the crude extract by storage at 2° for 2 weeks does not result in loss of inhibition. As mentioned previously the Kᵢ value for indolepyruvate does not change as the enzyme is purified. Therefore, it is not likely that the loss of inhibition by the competitive inhibitors, L-tryptophan and indolmycin is due to a conformational change at the active site during purification. A third possible

| Compound added            | Concentration | Enzyme activity |
|---------------------------|---------------|-----------------|
| p-Chloromercuribenzoate    | 0.005         | 42              |
| 0.01                      | 3             |
| p-Chloromercuribenzoate    | 0.01          | 99              |
| + 1.0 mM cysteine         |               |                 |
| N-Ethylmaleimide          | 4.0           | 48              |
| Iodoacetic acid           | 1.0           | 81              |
| Iodoacetamide             | 2.0           | 56              |
|                           | 0.5           | 102             |
|                           | 1.0           | 107             |
|                           | 2.0           | 94              |
The presence of the tryptophan transaminase and the indolepyruvate C-methyltransferase in cell-free extracts of *Streptomyces griseus* supports the hypothetical pathway for indolmycin biosynthesis proposed by Hornemann et al. (7) in which tryptophan is transaminated first to indolepyruvate which is subsequently methylated at position 3 of the aliphatic side chain to form 3-methylindolopyruvate. An alternative pathway could be postulated which would involve C-methyltransferase activity at the stage of tryptophan rather than indolepyruvate. Previous feeding experiments had shown that 3-methyltryptophan was incorporated efficiently into indolmycin, suggesting that this compound could be an intermediate in the pathway (7). However, in unpublished experiments 3-methyltryptophan formation could not be detected unequivocally in the culture by trapping experiments. This fact combined with the finding that tryptophan is not methylated by the crude cell-free extract indicates that 3-methyltryptophan is not a natural intermediate in the biosynthesis. The finding that the transaminase is able to convert 3-methyltryptophan to 3-methylindolopyruvate explains the observed incorporation of exogenous 3-methyltryptophan, although this route is apparently not followed naturally.

Aromatic amino acid transaminases are fairly widespread in nature but most of those that have been purified and characterized transfer the methyl group of S-adenosylmethionine to oxygen, nitrogen, or sulfur atoms (37). The C-methyltransferase we have isolated resembles most other methyltransferases that utilize S-adenosylmethionine in that no cofactors are required for the reaction to occur. One exception is the Δ^4*-sterol methyltransferase reported by Moore and Gaylor (12) and by Bailey et al. (38) which requires glutathione for maximum activity. Most other S-adenosylmethionine methyltransferases so far reported have no requirement for divalent metal ions. A few (11, 39) however, do require Mg^{2+} for maximum activity and at least one other methyltransferase, a homocysteine S-methyltransferase, has been reported to be a metalloenzyme, most likely containing Zn^{2+} (40).

The observed inhibition of the indolepyruvate C-methyltransferase by p-chloromercuribenzoate and N-ethylmaleimide suggests that a sulfhydryl group is necessary for activity. Sulfhydryl groups are essential for the activity of a number of other methyltransferases (41, 42) but in no case is it known whether their role is in maintenance of enzyme conformation or in catalysis.

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The K_m value established for S-adenosylmethionine (1.3 \times 10^{-4} M) is comparable to the values observed for many O-, N-, and S-methyltransferases (43, 44) and for the DNA-C-methyltransferase from *E. coli* (45). The high affinity of the enzyme for indolepyruvate (K_m = 4.8 \times 10^{-8} M) may reflect the need to prevent build-up of high intracellular concentrations of this unstable substrate.

The significance of the observed inhibition of C-methyltransferase activity by indolmycin and L-tryptophan in the crude enzyme extract is unclear, especially since this property is lost upon purification of the enzyme. The inhibition by indolmycin might be attributed to feedback inhibition by the
end product. It is a commonly observed phenomenon in primary metabolic pathways that the first pathway-specific enzyme is under feedback control and there is some evidence of feedback inhibition occurring also in the biosynthesis of secondary metabolites. For example, chloramphenicol production is inhibited by concentrations of the antibiotic which are not toxic to the growth of the producing organisms, Streptomyces venezuelae (46, 47). Also, the first enzyme in ergot alkaloid biosynthesis, dimethylallyltryptophan synthetase, appears to be inhibited by end products of the pathway (48). Indolmycin, however, is found largely in the culture medium and does not cause significant inhibition of the enzyme activity. It appears to be inhibited by end products of the pathway (48).

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