Oxidation of Indan to Indanol and Indanone by the Hepatic Microsomal System*

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ABSTRACT: Indan is oxidized in two steps to a mixture of 1-indanol and 1-indanone by a rat liver microsomal preparation.

The first step is a typical microsomal oxygenation. The indanol formed is predominantly the (*S*)-(+) isomer. The second step, the dehydrogenation of indanol is catalyzed by a microsomal-bound oxidoreductase. This enzyme is stereoselective for (*S*)-(+)-indanol. Indanol dehydrogenase activity is also found in the soluble fraction and is not identical with liver alcohol dehydrogenase.

Studies on the mechanism and stereochemistry of the microsomal hydroxylation of ethylbenzene have recently been reported (McMahon *et al.*, 1969b). In those studies the hydroxylation of ethylbenzene to methylphenylcarbinol was shown to occur by a front-side displacement of hydrogen by the oxygen atom. Molecular oxygen served as the source of oxygen.

These studies on the nature of microsomal aliphatic hydroxylation have now been extended to include an investigation of the oxygenation of indan. In preliminary studies (Billings *et al.*, 1968) it was found that although 1-indanol was formed, as expected, 1-indanone was also an important product. This result was reminiscent of a number of other cases in which the oxidation of a foreign compound has been reported to occur in two steps, *i.e.*, hydroxylation followed by dehydrogenation to the corresponding carbonyl compound. Examples include the microsomal oxidation of nicotine to cotinine (Hucker *et al.*, 1960), the *in vivo* oxidation of hexobarbital (*cf.* Bush and Sanders, 1967), and the conversion of tremorine into oxytremorine (Hammer *et al.*, 1968).

Thus a study of microsomal oxidation of indan seemed of particular interest since it should lead not only to new information concerning aliphatic hydroxylation but also to a better understanding of the coupled conversion of carbinol into ketone.

Experimental Section

Materials. Indan, indanol, and indanone were obtained commercially and purified to >98% purity by chromatographic procedures. Pyridine nucleotides and isocitric dehydrogenase were purchased from C. F. Boehringer and Soehne. Glusulase, a combination of glucuronidase and sulfatase, was obtained from Endo Laboratories.

Enzyme Preparation. Livers were removed from 300-g male rats (Purdue–Wistar strain) that had been sacrificed by decapitation. The livers were immediately homogenized in 4 volumes of 0.1 M potassium phosphate buffer (pH 7.4) in a Potter–Elvehjem homogenizer at 0° using a Teflon pestle. The homogenate was centrifuged at 15,000*g* for 30 min and the supernatant containing the soluble plus microsomal fraction, was removed. The 15,000*g* supernatant was then recentrifuged at 105,000*g* for 45 min at 0°. After discarding the supernatant the microsomal pellet was resuspended in an equal volume of 0.1 M potassium phosphate buffer and the suspension was recentrifuged for 45 min at 105,000*g*. The microsomes were then suspended in sufficient 0.1 M phosphate buffer to yield a solution containing microsomes equivalent to 0.4 g of liver/ml. For induction experiments, microsomes were prepared in the same fashion from livers from rats pretreated with sodium phenobarbital (40 mg/kg per day by intraperitoneal administration) for 4 days and sacrificed on day 5.

Incubations. Microsomal fraction, prepared as described above, was used as the source of enzyme in all of the incubations. The specific reaction conditions used in each experiment are summarized in the footnotes to the tables and figures in the Results section. The microsomal incubations were terminated by the addition of 2 ml of 10% $ZnCl_2$ solution. The solution was then treated with 1 ml of saturated $Ba(OH)_2$ solution. After removal of precipitated protein by centrifugation, the reaction products were extracted into chloroform.

Gas–Liquid Partition Chromatography. Indanone, indanol, and indan were separated and quantitated by gas–liquid partition chromatography. The analyses were performed with an F and M Biomedical Model 402 gas-liquid chromatograph employing a 4-ft glass column, optical density 3 mm o.d. The column, which was packed with 3.8% W98 (Silicone gum rubber) on a Diaport S support, was kept at a temperature of 120°. The hydrogen-flame detector was operated at 135°. For the separation of tetralol and tetralone a column packed with 15% HiEff-1BP was used. The column temperature was 190° and that of the detector, 205°.

Optical Activity Measurement. The optical rotations of indanol samples were determined in methanol in a 1-dm micropolarimeter tube of 0.2-ml volume.

Intact Animal Studies. The fate of indan, indanol, and indanone was also determined in the intact rat. Groups of six male, 200-g rats (Purdue–Wistar strain) were given 100 mg of indan/day per rat for 2 days. Urine was collected for 48 hr and pooled. The urine was adjusted to pH 5.5 with acetic acid, and 0.1 ml of Glusulase was added. After incubation

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TABLE I: Rate of Hydroxylation of Indan.[a]

Microsomes Used	μmoles of Indanol Formed in min		
	15	30	60
Control	1.3	1.9	1.9
Phenobarbital induced	7.1	9.2	10.7
DPEA (10^{-6} M) inhibited	0.9	0.9	0.8

[a] Conditions: Each flask contained microsomes from 1 g of liver, 1.4 μmoles of TPN^+, 50 μmoles of magnesium chloride, 25 μmoles of DL-isocitric acid, 10 mg of isocitric dehydrogenase, 300 μmoles of phosphate buffer (pH 7.4), and 160 μmoles of indan. The flasks were incubated with shaking in air for the specified length of time and then assayed for 1-indanol production.

for 18 hr at 37° the pH was adjusted to 7 and the metabolites were extracted into methylene chloride. The yield of indanol and indanone was determined by the gas–liquid partition chromatography procedure described above. Further similar studies were also carried out in which rats were dosed with either indanol or indanone.

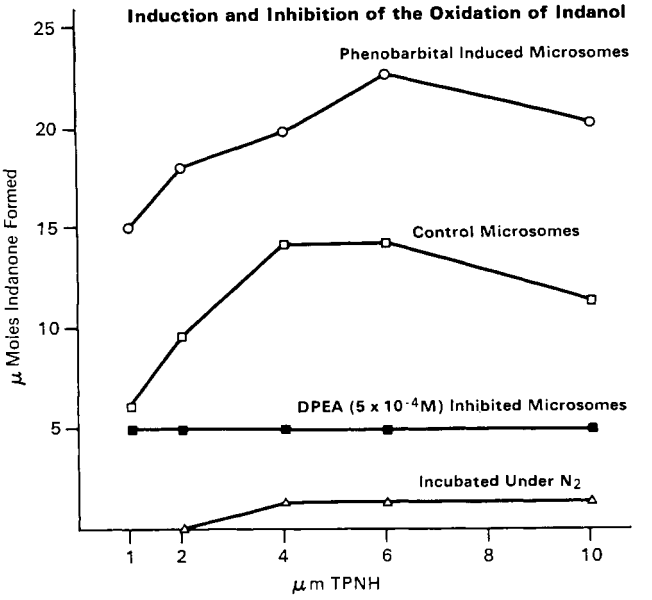

FIGURE 1: The formation of indanone from indanol. Each flask contained microsomes from 1 g of liver, 50 μmoles of nicotinamide, 75 μmoles of indanol, varying amounts of TPNH, and sufficient 0.1 M phosphate buffer (pH 7.4) to make a total volume of 3 ml. The flasks were incubated at 37° with shaking in air for 60 min and then assayed for 1-indanone.

Results

Indan Hydroxylation. In an initial experiment, 160 μmoles of indan was incubated for 1 hr in air with 1 μmole of TPNH and the microsomal fraction from 400 mg of liver. Under these conditions the product consisted of 0.4 μmole of indanol and 1.2 μmoles of indanone. The presence of indanone was additionally confirmed by the preparation of its 2,4-dinitrophenylhydrazone derivative.

In a second experiment, TPNH was replaced by a TPNH-regenerating system, isocitrate–isocitrate dehydrogenase. Under these conditions the rate of indanone formation relative to indanol was substantially suppressed. Thus in typical runs, indanol predominated over indanone in a ratio of 2–4:1. Because indanone production was lower in this system it was used to study the rate of hydroxylation of indan to indanol in control, phenobarbital-induced and DPEA[1]- (McMahon *et al.*, 1969a) inhibited microsomes. The results, which are presented in Table I, showed the reaction to be a typical hydroxylation, stimulated by phenobarbital pretreatment and inhibited by DPEA.

To determine the stereospecificity of the reaction, the hydroxylation of indan with control microsomes and TPNH-generating system was carried out on a scale 25 times greater than that used for the analytical runs. From this reaction, 11.8 mg of 1-indanol and 6.3 mg of 1-indanone were obtained. Polarimetry measurements showed the carbinol to be racemic. The preparatory scale run was repeated using a 15,000*g* liver supernatant as a source of enzyme instead of microsomal pellet material. This run produced 14.1 mg of 1-indanol and 3.6 mg of 1-indanone. The carbinol was found to have $[\alpha]_D^{25}$ (+)17°, *c* 0.7, methanol. Based on the known rotation of optically pure indanol and the known absolute configuration of the indanols (*cf.* Brewster and Buta, 1966) this corresponds to a mixture of 76% (*S*)-(+)-indanol and 24% (*R*)-(−)-indanol.

In yet another run in which 15,000*g* supernatant was used as an enzyme source the product consisted of 26.7 mg of indanol and 14.6 mg of indanone. In this case the indanol had $[\alpha]_D^{25}$ (+)7.7°, *c* 1.1, methanol. This corresponds to a mixture of 62% (*S*)-(+)-indanol and 38% (*R*)-(−)-indanol.

Indanol Dehydrogenation. The dehydrogenation of indanol to indanone was next investigated. For example, the extent of indanone formation from indanol at increasing concentrations of TPNH was determined under various conditions. These results are shown in Figure 1. With control microsomes good yields of indanone resulted. The yield increased with increasing TPNH concentration up to 4 μmoles beyond which the rate did not increase. When microsomes from phenobarbital-treated rats were used the rate of indanone formation was substantially increased over control values. On the other hand, the reaction was partially suppressed by DPEA as well as by incubation under nitrogen. Thus the oxidation of indanol to indanone by microsomes in the presence of TPNH has many of the characteristics of a typical microsomal oxygenation. However, the stoichiometry of the reaction argued against this interpretation. For example, in the presence of 4 μmoles of TPNH about 14 μmoles of indanol was formed with control microsomes. A maximum of 1 mole of oxygenated product from each mole of TPNH would have been expected in the microsomal oxygenase system. That the reaction is not a TPNH–O_2-dependent microsomal oxygenation was confirmed when it was found that TPN^+ could completely replace the requirement for TPNH (see Figure 2). Even DPN^+ partially replaced TPNH.

[1] The abbreviation used is: DPEA, 2,4-dichloro-6-phenylphenoxyethylamine.

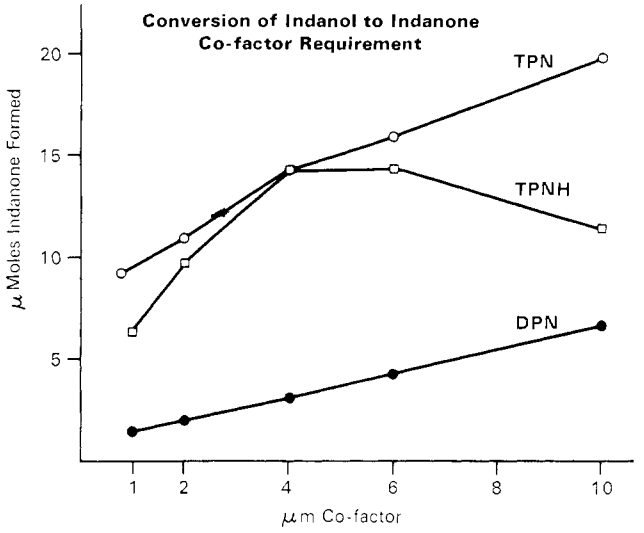

FIGURE 2: Cofactor requirement for the conversion of indanol into indanone. Conditions were the same as in Figure 1 except that TPNH was replaced by TPN^+ or DPN^+.

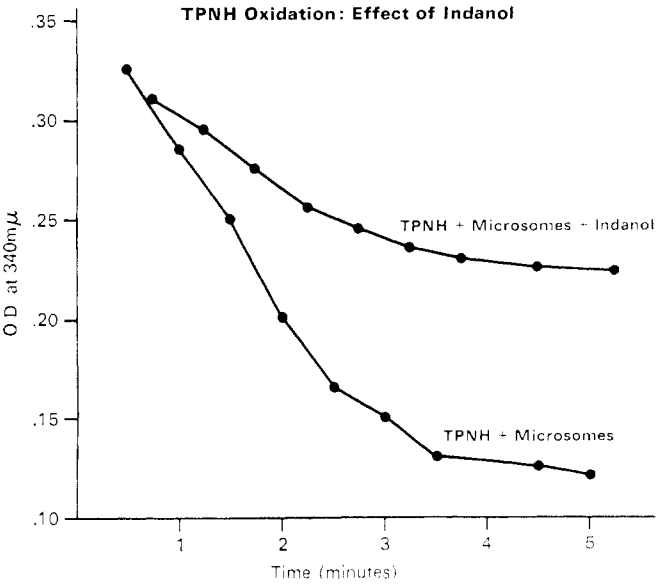

FIGURE 3: The effect of indanol on microsomal TPNH oxidation. Each absorbance cell contained microsomes from 300 mg of liver, 50 μmoles of magnesium chloride, 50 μmoles of nicotinamide, 0.2 μmole of TPNH, and 300 μmoles of phosphate buffer (pH 7.4). Measurements were made in a Shimadzu MPS-50L spectrophotometer.

The effect of indanol on the apparent rate of oxidation of TPNH to TPN^+ by microsomal TPNH oxidase (Gillette *et al.*, 1957) was also studied. The results, shown in Figure 3, demonstrate that in the presence of indanol TPNH oxidation is slowed, presumably because TPNH is being regenerated in the indanol dehydrogenation step.

It was also found that the indanol dehydrogenation system could serve as a TPNH-generating system for a typical microsomal drug oxidation, the demethylation of D-propoxyphene (McMahon, 1961). Thus the addition of indanol to a mixture of D-propoxyphene, TPN^+, and microsomes resulted in a 17-fold increase in demethylation rate over control rates in the absence of indanol.

Intracellular Distribution of Indanol Dehydrogenase Activity. The question of whether indanol dehydrogenase activity is associated exclusively with the microsomal fraction was also investigated. It was found that a 100,000*g* supernatant fraction from the 15,000*g* fraction of liver homogenate readily oxidized indanol in the presence of either DPN^+ or TPN^+. The activity present in the soluble fraction was apparently not, however, alcohol dehydrogenase. Horse liver alcohol dehydrogenase did not catalyze the oxidation of indanol to indanone in the presence of either TPN^+ or DPN^+ under any of a variety of pH values and reaction conditions. Attempts to remove more of the activity from the microsome fraction by repeated extraction with buffers were without success.

Stereoselectivity of Indanol Dehydrogenation. A preparatory scale (25 times the analytical scale) run was made in which racemic indanol was dehydrogenated in the presence of microsomes and TPN^+. From 70 mg of indanol were obtained 23.6 mg of indanone and 25.6 mg of purified recovered indanol. The recovered indanol had $[\alpha]_D^{25}$ (−)17.1° equivalent to a mixture of 75% (*R*)-(−)-indanol and 25% (*S*)-(+)-indanol showing that the reaction is stereoselective for the (*S*)-(+) isomer.

Tetralol as Substrate. It was found that 1-tetralol, the six-membered ring analog of indanol, was also readily dehydrogenated by the microsomal system. Thus when 75 μmoles of tetralol was incubated with microsomes from 1 g of liver and 5 μmoles of TPN^+, 6.5 μmoles of tetralone was formed in 30 min. Under these conditions 7.2 μmoles of indanone was obtained from 75 μmoles of indanol. A repetition of the experiment in which 5 μmoles of TPNH was substituted for TPN^+ yielded 6.1 μmoles of tetralone. Tetralol was also readily dehydrogenated by the soluble fraction with either TPN^+ or DPN^+.

The Fate of Indane, Indanol, and Indanone in the Intact Rat. The *in vivo* fate of indan, indanol, and indanone in the rat was investigated. Results are summarized in Table II. Following administration of indan to rats, about 4% of the dose was recovered as carbinol (or conjugated carbinol) and <0.2% as ketone. When indanol was given, 3% appeared in urine as indanol (or its conjugate) and <0.1% as indanone. Finally when indanone was given neither carbinol nor ketone was found in urine.

Discussion

The most significant result of this study of the oxidation of indane by liver microsomes was that substantial amounts of ketone could be formed in addition to the expected carbinol. The data presented above can best be interpreted to mean that indan is first hydroxylated to indanol which is in turn dehydrogenated to ketone in a second step (summarized in Figure 4).

The first step appears to be a typical microsomal hydroxylation. For example, the rate of the reaction is increased by the use of microsomes prepared from rats that have been pretreated with phenobarbital. Conversely the reaction is inhibited by the addition of a microsomal inhibitor, DPEA (see Table I).

It was not possible to determine the stereoselectivity of the

TABLE II: *In Vivo* Metabolism Studies.

Substrate[a]	% of Dose Recovered As	
	Indanol	Indanone
Indan	4.1	0.15
Indanol	3.0	0.08
Indanone	0.0	0.0

[a] The dose in each instance was 100 mg/rat per day to six rats (total 1200 mg).

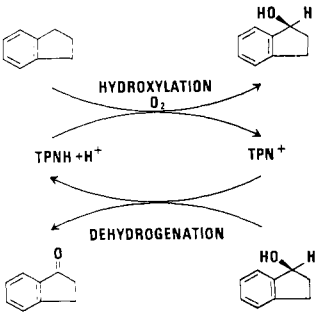

FIGURE 4: The conversion of indan into (*S*)-(+)-indanol and indanone in rat liver microsomes.

hydroxylation reaction directly since a system could not be devised in which the indanol produced was not oxidized in part to indanone. However the steric bias of the reaction could be inferred. Thus in each of three runs the carbinol formed was either racemic or was predominantly (*S*)-(+)-carbinol, depending on how much indanone was formed concomitantly. Since the (*S*)-(+)-carbinol was the preferred substrate for indanone formation these results suggest that the indanol formed in the hydroxylation reaction is even more predominantly the (*S*)-(+)-carbinol than that which is isolated. The observation that the amount of (*S*)(+)-indanol in the product is inversely related to the amount of indanone formed is consistent with this interpretation.

Dehydrogenation of Indanol. The observations that the rate of conversion of indanol into indanone by microsomes required oxygen, was enhanced by phenobarbital pretreatment, and was inhibited by DPEA suggested that the reaction was indeed being catalyzed by a mixed-function oxidase. However, the stoichiometry of the reaction and the fact that TPN$^+$ could completely replace TPNH as cosubstrate showed this not to be true. Rather, the results are best explained by a cyclic reaction process in which TPNH is first oxidized to TPN$^+$ by microsomal TPNH oxidase. In the second step then, indanol is dehydrogenated to indanone by a microsomal bound oxidoreductase with the result antregeneration of TPNH. The requirement for oxygen, the effect of phenobarbital pretreatment and inhibition by DPEA are thus understandable as effects upon the TPNH oxidase reaction and not directly upon the dehydrogenation reaction itself. The suggestion that indanone is formed by a dehydrogenation involving reduction of TPN$^+$ to TPNH is further confirmed by the demonstration that this reaction can serve as a TPNH-generating system to support a typical microsomal mixed-function oxidation, the demethylation of propoxyphene. The effect of indanol upon the oxidation of TPNH by TPNH oxidase (Figure 3) is also consistent with this interpretation. That the yields of indanone are lower when DPN$^+$ is substituted as cofactor may not mean that it is a less effective cosubstrate but is rather a reflection of the absence of a DPN-regenerating system.

The nature of the enzyme responsible for indanol dehydrogenation and the role it plays in the metabolism of foreign compounds remains obscure. Preliminary results, however, suggest strongly that the enzyme is not identical with alcohol dehydrogenase. It may, however, be related to the soluble 3-hydroxymethylhexabarbital dehydrogenase described by Toki and Tsukamoto (1964). The cellular distribution experiment showed the activity to be present in both the microsomal fraction and the soluble fraction. However, it is not possible to say whether these are two different enzymes or whether a portion of the membrane bound dehydrogenase is solublized during the preparation of the microsomal fraction.

As expected, the enzyme showed a stereochemical preference for the (*S*) isomer. This is the isomer that is formed in the hydroxylation step and accounts for the high yield of ketone from hydrocarbon in the absence of a TPNH-generating system.

Tetralol was dehydrogenated almost as rapidly as indanol by both the microsomal system and by the soluble fraction. Thus it is surprising that recent studies (Chen and Lin, 1968) of the oxidation of tetralin by a 10,000*g* liver supernatant fraction containing microsomes yielded only carbinol instead of carbinol plus ketone. In another related study, Drummond and Hopkins (1969) have reported the dehydrogenation of *trans*-acenaphthene-1,2-diol, by rat liver microsomes. Also Sims (1966) reported that one of the pathways in the metabolism of methylcholanthrene in microsomes involves the dehydrogenation of 1- and 2-hydroxy-3-methylcholanthrene. Although indanol, tetralol, acenaphthenediol, and the two hydroxycholanthrenes are closely related alcohols they have no obvious relation to any natural liver substrate. Thus the question of the natural substrate for this enzyme remains obscure.

In an effort to relate the results of this study to the intact rat, the *in vivo* fate of indan, indanol, and indanone was briefly investigated. It is clear from the results (Table II) that all three of these compounds are metabolized for the most part to unidentified metabolites. The fact that the yield of carbinol from hydrocarbon is low does not necessarily mean that this pathway is unimportant since the carbinol itself when administered undergoes further metabolism. Likewise the absence of ketone formation from hydrocarbon or carbinol cannot be evaluated since the ketone appears to be completely metabolized to other compounds when it is administered. These results are reminiscent of those obtained with ethylbenzene (McMahon and Sullivan, 1968), which is metabolized in part to (*S*)-mandelic acid in a reaction sequence involving a number of intermediates including carbinol and ketone. Similarly a thorough study of the fate of tetralin in the rabbit (Elliott and Hanam, 1968) shows it to be metabolized to a variety of oxygenated metabolites. With tetralin the α-carbinol is a major metabolite and as in the case of indan no corresponding ketone was found.

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Nucleoside Antibiotics. VI. Biosynthesis of the Pyrrolopyrimidine Nucleoside Antibiotic Toyocamycin by *Streptomyces rimosus**

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ABSTRACT: The biosynthesis of the pyrrolopyrimidine nucleoside antibiotic, toyocamycin elaborated by *Streptomyces rimosus*, has been studied. Adenine-2-^{14}C, but not adenine-8-^{14}C, is incorporated into toyocamycin. All of the ^{14}C in the toyocamycin from the adenine-2-^{14}C experiments resides in C-2 of toyocamycin. This was shown by the conversion of toyocamycin (**1**) into the 3-carboxyethyl derivative (**5**). C-2 of toyocamycin was released as formic acid by heating **5** in alkali. 5-Carboxy-4-hydroxy-7-(β-D-ribofuranosyl)pyrrolo-[2,3-*d*]pyrimidine (**8**), synthesized from the intermediate 2-amino-4-carboxy-3-carbonyl-[*N*-(2-carboxyethyl)]-1-β-D-ribofuranosylpyrrole (**7**), by treatment with formic acid and acetic anhydride was not radioactive. Compounds **2**, **3**, **4**, **5**, and **8** were all isolated and crystallized, and their structures were rigorously characterized. The isolation of formic acid (**6**) from **5** was unequivocally established. These data provide evidence that N-7 and C-8 of the imidazole ring of a purine are lost during the biosynthesis of the pyrrole ring of toyocamycin. 5-Carboxamido-4-hydroxy-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine (**3**) has been alkylated in good yields with β-propiolactone at N-3 of the pyrrolopyrimidine ring to form the carboxyethyl nucleoside (**5**). This procedure affords an excellent method for opening of the pyrimidine ring and the isolation of C-2 as formic acid. The studies reported here add another role of purines in the biosynthesis of naturally occurring compounds.

During the past 18 years 34 nucleoside antibiotics, having a wide spectrum of chemical structures and biological activity against viruses, bacterial, and tumor cells, have been isolated from the bacteria and fungi (Suhadolnik, 1970). The importance of adenine and/or adenosine in the biosynthesis of the nucleoside antibiotics, cordycepin (3′-deoxyadenosine), 3′-amino-3′-deoxyadenosine, 3′-acetamido-3′-deoxyadenosine, psicofuranine, decoyinine, tubercidin, and toyocamycin have already been reported from this laboratory (Suhadolnik, 1970).

The three known pyrrolopyrimidine nucleoside antibiotics, tubercidin, toyocamycin (**1**), and sangivamycin (**2**) have been isolated from the culture filtrates of *Streptomyces* in eight independent laboratories. Tolman *et al.* (1968) established the chemical structure of tubercidin, toyocamycin, and sangivamycin. The synthesis of toyocamycin by these investigators proved that toyocamycin, unamycin B, vengicide, and antibiotic E-212 have the same structures.

This laboratory had reported earlier that ^{14}C from adenine-2-^{14}C, but not adenine-8-^{14}C, was incorporated into the pyrrolopyrimidine ring of tubercidin and toyocamycin (Smulson and Suhadolnik, 1967; Suhadolnik and Uematsu, 1968). These isotope-labeling patterns suggest that the pyrimidine ring of a purine was used directly in the biosynthesis of the pyrimidine

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