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COMPARATIVE METABOLISM OF ETHAMBUTOL AND ITS L-ISOMER

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Abstract—The absorption, excretion, and metabolism of the D- and L-isomers of ^{14}C-labeled 2,2′-(ethylenediimino)-di-1-butanol have been studied. Both isomers disappear rapidly from the plasma after i.v. or oral administration in the dog, with the D-isomer being removed more rapidly from the circulation than the L. The drug accumulates in or on the erythrocytes. Differences in the excretion of the two isomers also exist; 97% of an intravenous dose of the D-isomer is recovered from the excreta as compared with 81% of the dose of the L-isomer, Both isomers are metabolized by the dog to two compounds: (1) an intermediate metabolite aldehydic in nature and (2) a dicarboxylic acid -2,2′-(ethylenediimino)-di-butyric acid corresponding to the terminal oxidation product of the parent compound. The extent of metabolism of the L-isomer is greater than that of the D in the dog. Alcohol dehydrogenase and the soluble and microsomal fractions of rat liver convert the drug to an aldehyde, similar in nature to the primary metabolite.

THE antituberculosis activity of N,N′-diisopropylethylenediamine prompted studies of its analogues and resulted in the synthesis of 2,2′-(ethylenediimino)-di-1-butanol.[1] This compound is a racemic mixture of D-, L-, and small quantities of the *meso*-isomers. *In-vivo* studies of the stereoisomers in mice indicated that at the maximal tolerated doses the L form of the compound was inactive, that the D-isomer accounted for the greater amount of the antituberculosis activity, and that the *meso* form possessed only slight activity.[2] Ethambutol is the generic name that has been given the active dextrorotatory isomer of 2,2′-(ethylenediimino)-di-1-butanol.

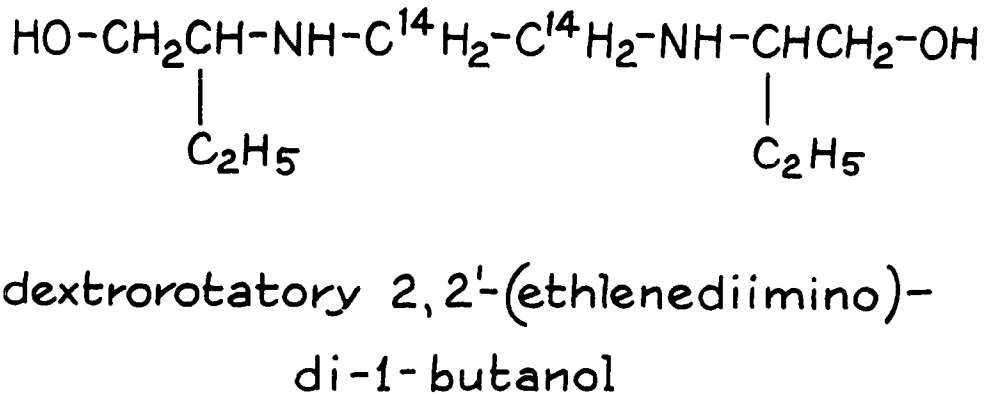
In the rhesus monkey ethambutol was shown to have antimycobacterial effects on established pulmonary tuberculosis and when administered with isoniazid blocked the emergence of isoniazid-resistant bacilli.[3] The utility of this compound in the established human disease is now under study, and the clinical pharmacology and metabolism of the compound in such patients have been reported.[4–6]

Because of the possible usefulness of ethambutol in the treatment of tuberculosis and its stereochemical requirement for activity, it was of interest to study the pharmacokinetics and metabolism of this drug and to compare some of these properties with those of the inactive L-isomer.

METHODS

Ethambutol and the L-isomer specifically labeled with ^{14}C were used throughout these experiments and were administered as the dihydrochloride salt. The terms ethambutol and L-isomer are used to designate the dextro- and levorotatory isomers of the

compound, respectively. The radioactive compounds were prepared from uniformly labeled ethylene dibromide and dextro- or levorotatory 2-aminobutanol.[7] Figure 1 shows the structure of ethambutol and the positions of the ^{14}C label. The specific activity of ethambutol was 1·02 μc ^{14}C/mg and that of the L-isomer was 0·98 μc ^{14}C/mg. Purity and radiohomogeneity of these labeled compounds were determined by comparative paper chromatography in several solvent systems, countercurrent distribu-



dextrorotatory 2,2'-(ethlenediimino)-di-1-butanol

FIG. 1. Ethambutol, specifically radiolabeled.

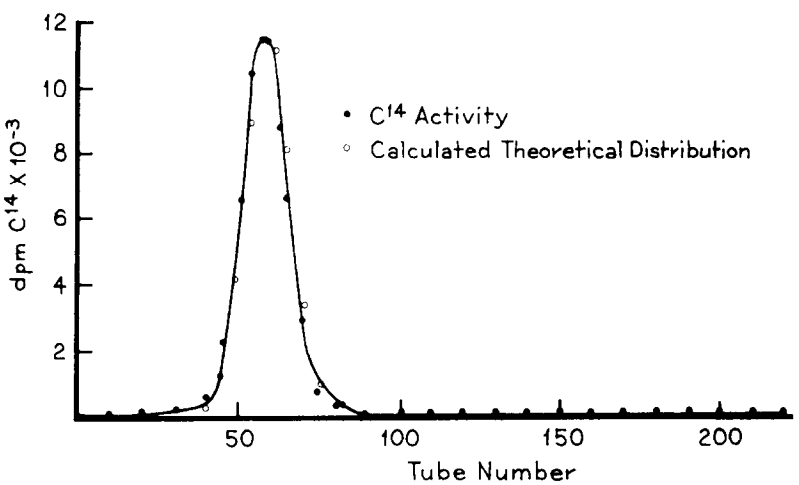


FIG. 2. Countercurrent distribution of ^{14}C specifically labeled ethambutol. Solvent system, *n*-butanol: acetic acid:water (4:1:5); 303 transfers.

tion, and microbiological assay of ethambutol. Countercurrent distribution of labeled ethambutol resulted in a single peak of radioactivity of theoretical proportions (Fig. 2). Similar results were obtained in a distribution of the L-isomer. Samples removed from the peak tube of the countercurrent distribution of either isomer moved as a single component on paper chromatograms and had an R_f in exact agreement with

that of authentic compound. Moreover, biochromatograms indicated that the radioactive area of the chromatograms of ethambutol coincided exactly with the area having microbiological activity.

The dogs used in this study were pedigreed male beagles of 8–10 kg from our colony. In studies in which the pharmacology and metabolism of ethambutol and the L-isomer were compared, the same dog was used in order to minimize biological variation within a species. The dogs received into the leg vein an i.v. injection of 50 mg of drug/kg, dissolved in isotonic saline, or an oral dose of 100 or 200 mg/kg in a single gelatin capsule. In all cases the radiolabeled preparation was diluted with non-labeled drug to give the desired specific activity.

After drug administration the animals were confined to metabolism cages, and the urine samples were immediately frozen upon collection. Catheterized urine collections were made during the first hours following drug administration. Blood samples were withdrawn from the jugular veins of the unanesthetized dogs into heparin-coated syringes at intervals which ranged from 15 min to 72 hr after drug administration.

Radioactivity measurements were performed in the Packard Tri-Carb liquid scintillation spectrometer. The polyether-611 system[8] was used in the counting of urine and other aqueous samples, and materials soluble in organic solvents were counted in toluene phosphor. Plasma samples were first solubilized in 3 ml of Hyamine hydroxide and then distributed in toluene phosphor.[9]

All radioactivity measurements are expressed in terms of absolute units, which were determined by means of an internal standard technique. The counting efficiencies of countercurrent distribution fractions, however, were consistent enough to enable plots to be made of relative instead of absolute activity. For example, the average counting efficiency of 50 tubes of a typical distribution of a 24-hr total urine residue was 58·7 $\pm$ 1·2%, as determined by the internal standard method.

Radioactivity of solid samples such as whole blood and feces homogenates, and of samples too large to be counted directly, was determined by an oxygen-flask combustion technique.[10] The zones of radioactivity on paper chromatograms were detected in the radiochromatogram scanner (Atomic Accessories, Inc., model RSC160) or by counting sections of the chromatogram in the liquid scintillation counter.[11]

Countercurrent distribution analysis of raw urine samples was performed either by charging the apparatus with aliquots of raw urine or by lyophilizing pooled collections and placing this residue, dissolved in the aqueous phase of the distribution system, into the apparatus. The countercurrent distributions were carried out in a 2 cc per phase 220 tube, or in a 12·5 cc per phase 200 tube automatic apparatus, and the solvent systems employed were an *n*-butanol:ammonium hydroxide:water (4:1:5) mixture or an *n*-butanol: acetic acid:water (4:1:5) system. The labeled components of the charge were withdrawn from the apparatus and the solvents removed by evaporation *in vacuo* and lyophilization, and the residues reserved for further study.

In our experience the movement of ethambutol, the L-isomer, or their transformation products in a countercurrent distribution was affected by the presence of urine salts, particularly when a solvent system was employed in which the compounds had low distribution coefficients. The consequence of this decrease or increase of the overall distribution coefficient of these diamines by the presence of substantial quantities of urine salts was a distribution peak characteristically narrower than the calculated

theoretical distribution. Because of these conditions, the countercurrent distribution coefficient of a material was never considered sufficient evidence for its identification. Instead, the components of a distribution were examined further by paper chromatography, comparisons being made to standard compounds before positive identifications were made.

Chromatograms were performed on Whatman 1 paper in the following systems: *n*-butanol:ammonium hydroxide or acetic acid:water (4:1:5); methanol:water:pyridine (20:5:1); phenol:water (4:1); isopropanol:ammonium hydroxide or formic acid or acetic acid:water (4:1:5); or amyl alcohol:5 M formic acid (1:1). In addition to radiochromatographic scanning, materials were located on paper by color-producing spray reagents which were a solution of 1% ninhydrin in *n*-butanol (w/v) previously equilibrated with McIlvaine's buffer, pH 7·0; a solution of 0·4% bromcresol purple in 95% ethanol (w/v) containing boric acid and aqueous borax solution; or an ammoniacal silver nitrate solution consisting of equal parts of 0·1 N silver nitrate and 0·1 N ammonium hydroxide mixed just prior to use.[12]

The microbiologically active materials on the chromatograms were located by the zone of inhibition technique with *Mycobacterium smegmatis* (ATCC 607) as a test organism.

The ability of alcohol dehydrogenase to effect the oxidation of ethambutol or its L-isomer was determined by incubating ethambutol, 10^{-2} M, with dilutions of the enzyme, in the presence of 10^{-4} M DPN, and 0·05 M phosphate buffer, pH 8·0. DPNH production was followed by measuring the absorption of the solution at 340 mμ in the Cary recording spectrophotometer. Incubations were carried out in a 30-ml quartz cuvette and measurements begun immediately upon the addition of the enzyme. Yeast and horse liver alcohol dehydrogenase were obtained from Mann Research Laboratories.

Studies of the metabolism of ethambutol by the soluble and microsomal liver enzymes were performed on fractions obtained from livers of male Sherman rats weighing about 250 g. The animals were decapitated and the livers rapidly removed and homogenized in a 0·25 M sucrose solution. The homogenate was centrifuged for 15 min at 9,000 g and the pellet discarded. The supernatant was then centrifuged at 105,000 g for 60 mins, yielding a microsomal pellet and a supernatant containing the soluble liver enzymes. The microsomes were washed twice with 0·25 M sucrose solution and suspended in 1 M phosphate buffer, pH 7·4. Microsomes equivalent to 3·4 g of liver, or soluble enzymes equivalent to 0·8 g of liver, or 3 ml of the whole homogenate were incubated with 500 μg of ^{3}H-labeled ethambutol in a solution containing 3 mg TPN, 14 mg glucose-6-phosphate, 0·25 ml $MgCl_2$ (0·5 M), and 6·8 enzyme units of glucose-6-phosphate dehydrogenase in a volume of 5 ml and the incubations conducted aerobically, with shaking, for 90 min at 37°.

In these experiments tritium-labeled ethambutol was used because it offered the advantage of a much higher specific activity (22 μc/mg) than that of the ^{14}C-labeled preparation, thereby enabling the detection of small amounts of transformation products. This material was prepared in our laboratory by the gas-labeling technique. The purity of this preparation was determined by methods similar to those described for the ^{14}C-labeled compound. Paper chromatography, countercurrent distribution, and chemical derivatization were used to detect and identify the products of the *in-vitro* metabolism of ethambutol by the various fractions.

RESULTS

Urinary and fecal excretion of ^{14}C after administration of ethambutol-^{14}C or the ^{14}C-L-isomer. Within 48 hr after an i.v. injection of ethambutol-^{14}C, about 90% of the radioactivity appeared in the urine, followed by trace quantities for several additional days. Approximately 1% of the dose appeared in the feces (Table 1).

The cumulative excretion of ^{14}C following two separate i.v. doses of the labeled L-isomer in the same dog, L6, is also shown in Table 1. As with ethambutol-^{14}C, most of the ^{14}C excretion was into the urine in the 48-hr interval following the dose. The total ^{14}C recovered in the excreta of the dog in these experiments, however, was 81% of the dose in 216 hr, as compared with a recovery of 97% of the ^{14}C of the dose of ethambutol.

In a second dog given similar i.v. injections of ethambutol and the L-isomer, lower excretion of the L-isomer was again observed; 81% of the injected ^{14}C of the L-isomer was recovered in the excreta in 192 hr, while 91% of the ^{14}C of ethambutol was recovered in the urine and the feces in a similar interval (Table 1).

Urinary excretion of ^{14}C after a single oral dose of radiolabeled ethambutol in the dog accounted for 98% of the dose in 72 hr and all the administered ^{14}C was recovered from the excreta in 216 hr (Table 1). The cumulative excretion of only 1% of the ^{14}C of the dose in the feces demonstrated the rapid and extensive absorption from the intestine of the dog.

Plasma and red blood cell concentrations of ^{14}C after the administration of ^{14}C-labeled ethambutol and the L-isomer. Plasma levels of ^{14}C after two i.v. injections of ethambutol or the L-isomer in the same dog are presented in Fig. 3. After the injection of the labeled ethambutol, a precipitous drop from the initial level of 40 μg ethambutol equivalents/ml of plasma occurred. At 2 hr the rate of removal of ^{14}C from the plasma became more gradual and at 24 hr, levels of ^{14}C equivalent to 2–3 μg of ethambutol/ml plasma remained. Calculations based on the initial concentration of ethambutol in plasma as determined by microbiological assay showed the apparent volume of distribution of drug to be 119% of the body weight, and it could be assumed, therefore, that the drug became concentrated in the cells within the body.

After the intravenous injection of the labeled L-isomer the initial ^{14}C plasma levels were the same as those occurring after the intravenous injection of ethambutol. After 1 hr, however, the plasma was cleared less rapidly of ^{14}C related to the L-isomer than it was of the radioactivity related to ethambutol; at 24 hr after the dose, levels of 4–5 μg equivalents of L-isomer/ml plasma remained. While generally lower in the initial interval, the plasma levels of ^{14}C following the intravenous administration of the D- and L-isomers into a second dog exhibited the same pattern, i.e. a more rapid clearance of the D-isomer.

Peak plasma levels of 55 μg ethambutol equivalents/ml plasma after an oral dose of 100 mg ethambutol-^{14}C-kg occurred approximately 1 hr after the dose. The disappearance of ^{14}C from the plasma after this time was quite rapid and followed the same pattern seen after an i.v. dose, and after 24 hr 17 μg of ethambutol/ml plasma remained (Table 2). Plasma radioactivity persisted through 96 hr.

In-vivo experiments (Table 2) demonstrated the rapid entry of the ^{14}C of radiolabeled ethambutol into the red blood cells. At 15 min after an oral dose of ethambutol-^{14}C in the dog, the amount of ^{14}C in the erythrocytes was greater by a factor of

TABLE 1. CUMULATIVE EXCRETION OF ^{14}C RADIOACTIVITY AS PER CENT OF ADMINISTERED DOSE AFTER THE ADMINISTRATION OF ^{14}C-LABELED ETHAMBUTOL OR L-ISOMER IN THE DOG

	Ethambutol-^{14}C			L-Isomer-^{14}C			Ethambutol-^{14}C		
	Dog L6,* 50 mg/kg i.v.			Dog L6,* 50 mg/kg i.v.			Dog L6 100 mg/kg p.o.		
Time (hr)	(% in urine)	(% in feces)	Total excretion %	(% in urine)	(% in feces)	Total excretion %	(% in urine)	(% in feces)	Total %
0–24	84·6(81·1–88·1)	0·5(0·1–0·8)	85·1	67·2(66·2–68·2)	0·4(0·3–0·5)	67·6	90·1	0·1	90·2
0–48	91·5(89·1–93·9)	1·2(1·2–1·2)	92·7	73·7(72·5–74·9)	2·3(1·9–2·6)	76·0	96·3	1·0	97·3
0–72	93·5(91·2–95·7)	1·3(1·3–1·3)	94·8	75·8(75·0–76·6)	2·5(2·1–2·8)	78·3	97·9	1·0	98·9
0–216	95·4(93·1–97·7)	1·3(1·3–1·3)	96·7	78·2(77·5–78·9)	2·5(2·1–2·8)	80·7	99·4	1·0	100·4
	Dog D15, 50 mg/kg i.v.			Dog D15, 50 mg/kg i.v.					
0–24	82·9	1·1	84·0	71·8	0·8	72·6			
0–48	86·3	1·3	87·6	75·7	1·5	77·2			
0–72	88·5	1·4	89·9	77·4	1·6	79·0			
0–192	89·8	1·4	91·2	79·6	1·6	81·2			

* Two separate injections were given this dog, and the average excretion per period and the range are indicated.

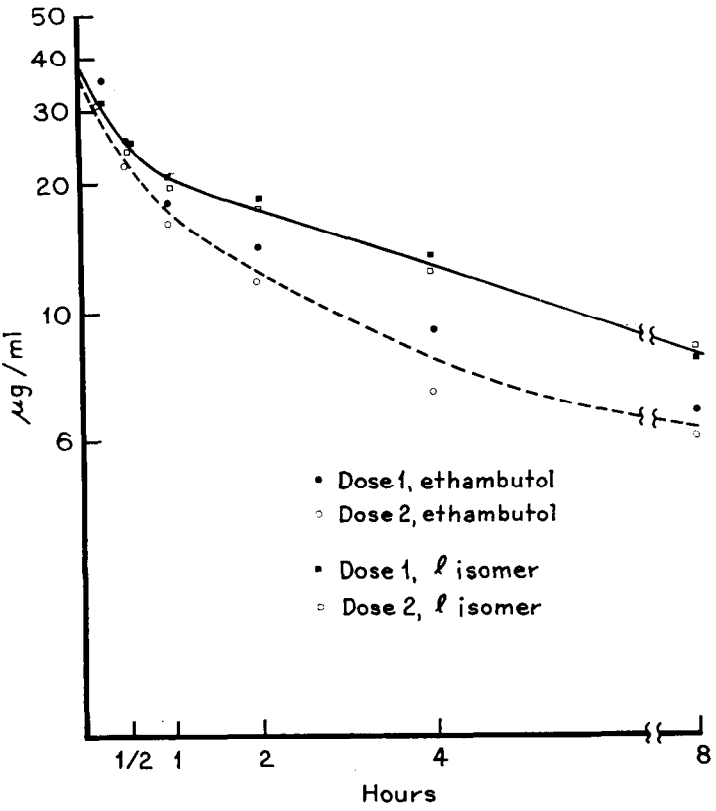


FIG. 3. Plasma levels of ^{14}C expressed as μg ethambutol or μg L-isomer after intravenous injection of the ^{14}C labeled drug in dog L6.

TABLE 2. PLASMA AND RED BLOOD CELL LEVELS* OF ^{14}C ASSOCIATED WITH ETHAMBUTOL OR THE L-ISOMER AFTER AN ORAL DOSE OF 100 MG/KG OR INTRAVENOUS INJECTION OF 50 MG RADIOLABELED DRUG PER KG

Time	Ethambutol, i.v. dose, Dog D15			Ethambutol, oral dose, Dog L6			L-Isomer, i.v. dose, Dog D15		
	Plasma (μg drug/ml)	Cells (μg drug/ml)	Cells/Plasma enrichment	Plasma (μg drug/ml)	Cells (μg drug/ml)	Cells/Plasma enrichment	Plasma (μg drug/ml)	Cells (μg drug/ml)	Cells/Plasma enrichment
15 min	23·2	35·0	1·5	2·6	12·0	4·6	28·3	37·2	1·3
30 min	16·1	56·1	3·5	18·5	37·2	2·0	19·8	31·0	1·6
1 hr	11·4	27·3	2·4	54·8	126·8	2·3	13·8	23·3	1·7
2 hr	7·3	23·2	3·1	29·0	85·5	2·9	10·6	22·1	2·1
4 hr	4·1	21·8	5·3	17·3	80·5	4·6	7·0	20·0	2·8
8 hr	1·7	18·5	10·9	11·7	61·0	5·2	4·3	19·1	4·4
24 hr	1·1	12·3	11·2	7·9	37·2	4·7	1·8	16·3	9·1

* The ^{14}C content of the cells is derived from measurement of radioactivity content of plasma and whole blood and of packed cell volume, as determined by using a Drummond microhematocrit apparatus.

$$^{14}C/\text{ml cells} = \frac{^{14}C/\text{ml blood} - [(1\text{-hematocrit})\ (^{14}C/\text{ml plasma})]}{\text{hematocrit}}$$

approximately 5 than that in the plasma. Between 15 min and 1 hr the ethambutol enrichment of the cells (^{14}C in cells/^{14}C in plasma) decreased to a value of 2, after which time the cell enrichment increased, and at 24 hr the cells contained again 5 times as much ^{14}C activity as the plasma. This condition could occur if the cells were cleared of ^{14}C faster and then slower than the plasma. Similarly, the erythrocytes became enriched in ^{14}C after an i.v. dose of ethambutol and the L-isomer. The enrichment at 24 hr following the injection of the D-isomer was 11; an erythrocyte enrichment of 9 occurred when the L-isomer was administered.

That the material in or on the erythrocytes was tightly bound was demonstrated by experiments in which ethambutol-enriched cells were washed with three successive portions of isotonic saline. This treatment removed only 50% of the material from the cells 15 min after dosing, and at 24 hr only 30% of the labeled material could be removed by similar treatment.

TABLE 3. PLASMA AND RED BLOOD CELL LEVELS OF ^{14}C AFTER THE *IN VITRO* INCUBATION* OF ETHAMBUTOL-^{14}C AND WHOLE DOG BLOOD

Time	^{14}C as μg ethambutol/ml		Cells/Plasma enrichment
	Plasma	Cells	
15 min	8·2	11·6	1·4
30 min	7·5	10·9	1·4
1 hr	6·3	14·2	2·3
2 hr	5·5	11·1	2·0
4 hr	4·1	12·8	3·2
20 hr	4·6	17·4	3·8

* Labeled ethambutol was added to freshly drawn oxalated whole blood and agitated in Dubnoff shaker under air at 37°.

In-vitro experiments also demonstrated the accumulation of ethambutol-^{14}C by the erythrocytes (Table 3). At 1 hr after the start of the incubation the cell/plasma enrichment was 2 and at 20 hr was 4. It was also of interest to note that while the accumulation was not as great as under conditions *in vivo*, after 4 hr of incubation little or none of the ^{14}C could be removed from the cells by repeated washing with isotonic saline.

Metabolic products of ethambutol-^{14}C and the radiolabeled L-*isomer.* Countercurrent distribution analysis had demonstrated the presence of two radiolabeled components in addition to the parent compound in the urine after the administration of labeled ethambutol or L-isomer in the dog. A variation of the urinary pattern of excretion of ^{14}C labeled compounds with time occurred after the i.v. injection of ethambutol-^{14}C (Fig. 4) and the injection of the ^{14}C-L-isomer (Fig. 5). These figures also show the distribution of ethambutol-^{14}C added to dog urine. The major peak possessed a distribution coefficient similar to that of ethambutol (or the L-isomer) when the parent compound was added to urine and distributed in a similar solvent system. Comparative paper chromatography further demonstrated that this peak was

unchanged ethambutol or the levorotatory isomer. Microbiological assay and biochromatograms in the case of ethambutol indicated that the material had continued biological activity.

Demonstrable amounts of metabolic products of ethambutol did not occur in the urine of the dog until 1 hr after the administration of the labeled drug (Fig. 4). At this time the two metabolites appeared, number I being the more polar and located closer to the charge tubes, and number II a transformation product of intermediate polarity. These metabolites persisted in the urine and at the end of 8 hr constituted approximately 4% and 5% of the administered radioactivity respectively. Unchanged parent was excreted in the amount of 57% of the dose.

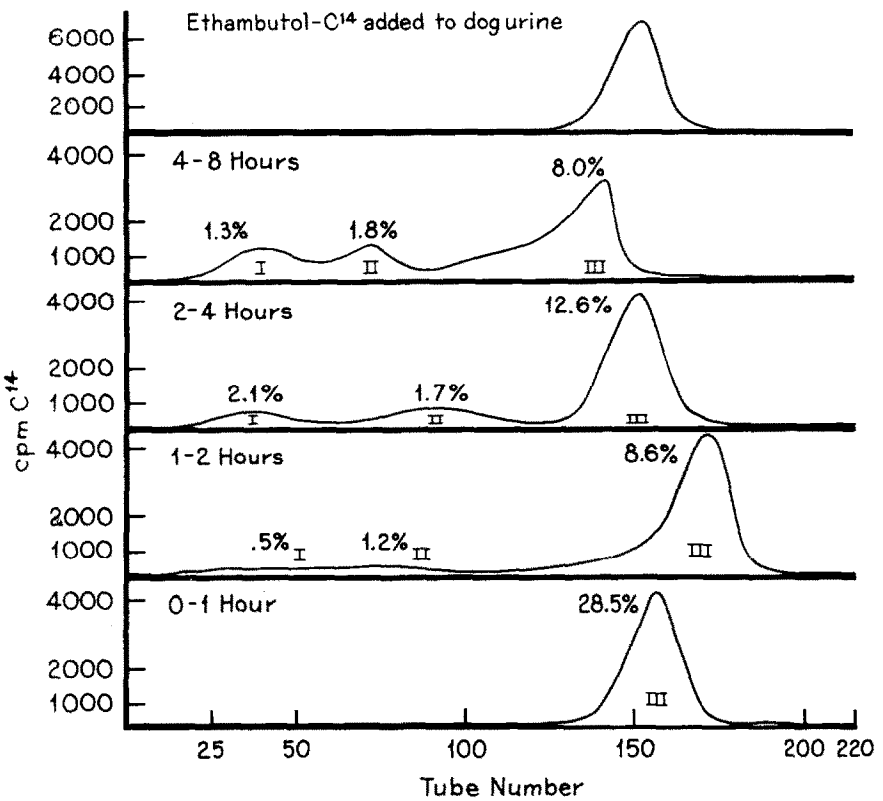


FIG. 4. Countercurrent distribution of aliquots of dog urine after the i.v. injection of ethambutol-^{14}C, showing ethambutol and metabolites as per cent of dose. Solvent system, *n*-butanol:ammonium hydroxide:water (4:1:5); 220 transfers.

In contrast to this was the urinary excretion pattern shown by the same dog given an i.v. injection of the labeled L-isomer (Fig. 5). The transformation products, I and II, appeared within the first hour after the dose and constituted a greater fraction of the administered radioactivity than did the metabolites of ethambutol in this same 8-hr period. Indeed, between 2–4 and 4–8 hours, the excretion of metabolites into the urine was greater than the excretion of parent compound. Through the 8-hr period,

metabolite I constituted approximately 14% of the dose; metabolite II, 10%; and unchanged parent, 31% of the dose.

The total urinary metabolic pattern through 48 hr following the i.v. administration of both radiolabeled isomers demonstrated that the greater portion of either isomer was excreted unaltered and that the metabolism of the L-isomer was at least twice greater than that of ethambutol (Fig. 6). Since 78% of the ^{14}C activity of the L-isomer was excreted in 48 hr, it could be calculated that 13% of the dose was excreted as metabolite I, 20% as metabolite II, and 45% as the parent. Similarly, of the 95% of the ^{14}C of ethambutol excreted into the urine, 5% was excreted as the metabolite I, 8% as II, and 82% as the unchanged parent compound. The metabolism of an oral dose of ethambutol-^{14}C was somewhat greater. In 24 hr after an oral dose of ethambutol-^{14}C, metabolites I and II amounted to 8% and 12% of the dose respectively, while 70% of the administered radioactivity was excreted as the unaltered parent.

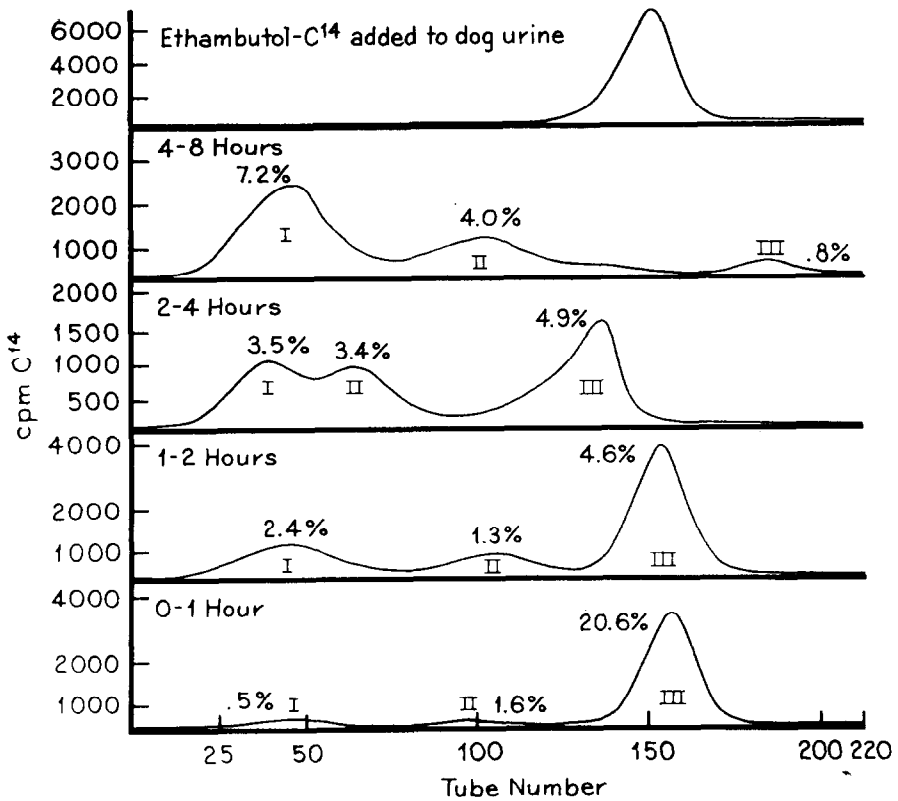


FIG. 5. Countercurrent distribution of aliquots of dog urine after the i.v. injection of ^{14}C—labeled L-isomer showing L-isomer and metabolites as per cent of dose. Solvent system, *n*-butanol: ammonium hydroxide:water (4:1:5); 220 transfers.

Investigations of the metabolism of ethambutol under conditions of chronic administration were also conducted. Two dogs were given daily oral doses of 100 mg ethambutol-kg for 30 days; on the first, seventh, and thirtieth day radiolabeled drug was given and the urinary metabolites measured as previously described. The results

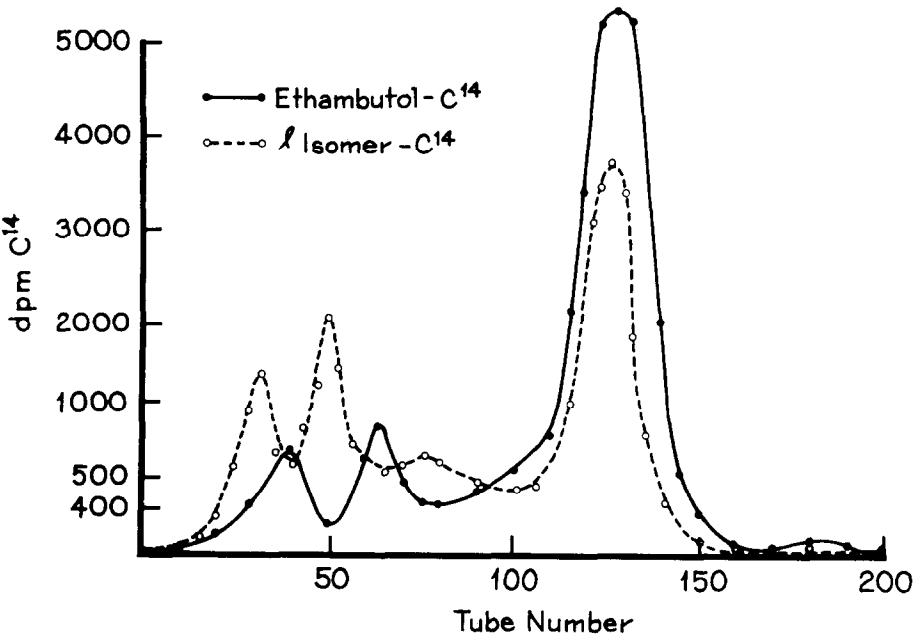


FIG. 6. Countercurrent distribution of radioactive material in the urine of the dog 0–48 hr after i.v. administration of radiolabeled ethambutol and L-isomer. Solvent system, *n*-butanol:ammonium hydroxide:water (4:1:5); 200 transfers.

of this study indicated that whereas no qualitative change in the metabolism of the drug occurred, there were changes in the extent of ethambutol transformation over a 24-hr period under this condition. In the 24-hr period following the initial dose of ethambutol the metabolism of the drug averaged 13% of the dose in the two dogs under study; metabolism of a similar dose after 30 days of daily administration averaged 24% of the dose (Table 4).

TABLE 4. THE METABOLISM OF ETHAMBUTOL-^{14}C IN THE DOG AFTER DAILY ORAL DOSES OF 100 MG/KG FOR 30 DAYS

		Ethambutol-^{14}C excretion and metabolism as % of dose/24 hr			
Dog	Day	Total excretion	Metabolite I	Metabolite II	Total metabolism
L6	1	57·9	5·3	7·0	12·3
L6	7	75·4	2·2	5·6	7·8
L6	30	78·8	7·7	15·8	23·5
JM	1	46·0	6·4	6·5	12·9
JM	7	81·7	9·5	12·9	22·4
JM	30	64·9	9·9	13·6	23·5

While sufficient data were not available to judge their statistical significance, these results were not inconsistent with the reports of drugs stimulating their own metabolism.[13]

Characterization of urinary metabolites. The material of high polarity present in peak I and that of intermediate polarity present in peak II of the countercurrent distribution were determined as being related to ethambutol (or the L-isomer) on the basis of radioactivity, which remained associated with a ninhydrin-positive material in several paper chromatographic systems. Upon treatment with the bromcresol purple spray reagent, the radioactive areas in similar chromatograms appeared as yellow spots, typical of acidic substances. The formation of gray-white spots of silver salts (a positive reaction of reducing or acid substances), when yet another series of chromatograms was sprayed with alkaline silver nitrate reagent, further indicated the acidic and/or aldehydic nature of the radioactive areas.

The dicarboxylic acid derivative of ethambutol, 2,2′-(ethylenediimino)-di-butyric acid, had been synthesized as a mixture of the D-, L-, and *meso*-isomers (M. Cantrall of these laboratories) and was compared chromatographically to the radiolabeled urinary metabolites. This synthetic compound migrated in four different paper chromatographic systems (Table 5) and in the countercurrent apparatus in a way very similar to that of the metabolite isolated from peak I.

TABLE 5. COMPARATIVE PAPER CHROMATOGRAPHY OF ETHAMBUTOL METABOLITES AND ITS DICARBOXYLIC ACID DERIVATIVE IN SOME TYPICAL CHROMATOGRAPHIC SYSTEMS*

Material	System I	System II	System III	System IV
Metabolite I	0·29 (streaked)	0·04	0·74	0·89
Metabolite II	streaked	0·54	0·82	0·95
2,2′-(Ethylenediimino)-di-butyric acid	0·29 (streaked)	0·04	0·74	0·88

* I *n*-Butanol: acetic acid: water (4:1:5).
II *n*-Butanol: ammonium hydroxide:water (4:1:5).
III Isopropanol: acetic acid:water (4:1:5).
IV Isopropanol:ammonium hydroxide:water (4:1:5).

The hydroxamic acid derivative of both metabolite I and the dicarboxylic acid was next prepared, first by esterification (by thionyl chloride–alcohol treatment or by refluxing with ethylene glycol and sulfuric acid) and then by treatment with hydroxylamine hydrochloride. When these two derivatives were chromatographed in two systems a single spot was detected upon spraying the chromatogram with ferric chloride. These spots were of the same R_f and were intense red-purple in color, a positive indication of a hydroxamic acid–ferric chloride complex. In the case of the metabolite I, all ^{14}C activity was located in this spot.

When subjected to esterification and hydroxamic acid derivatization via the same procedures, the metabolite of intermediate polarity, II, reacted positively (the reaction presumably proceeding through an oxidized form of this material if an aldehyde) and yielded a radioactive derivative having the same chromatographic mobility as the hydroxamate of metabolite I and of the authentic dicarboxylic acid derivative, and which formed a ferric chloride complex. These findings further indicated that the polar metabolite was 2,2′-(ethylenediimino)-di-butyric acid and that there was precursor–product relationship between the two metabolites.

The possibility that metabolite II might be an aldehyde was examined by subjecting a sample of dog urine which contained ethambutol-^{14}C and both metabolites to mild oxidation with Fehling's solution and to oxidation with 30% hydrogen peroxide. To aliquots of 4 ml of raw urine of a dog which had received an i.v. dose of ethambutol was added 5 ml of hydrogen peroxide, 30%, or 2 ml of Fehling's solution; the solution was allowed to stand for 48 hr when hydrogen peroxide was used or was heated for a few minutes at 100° when the oxidation with Fehling's solution was being conducted. The solutions were then introduced into the countercurrent apparatus and distributed as previously described (Fig. 7).

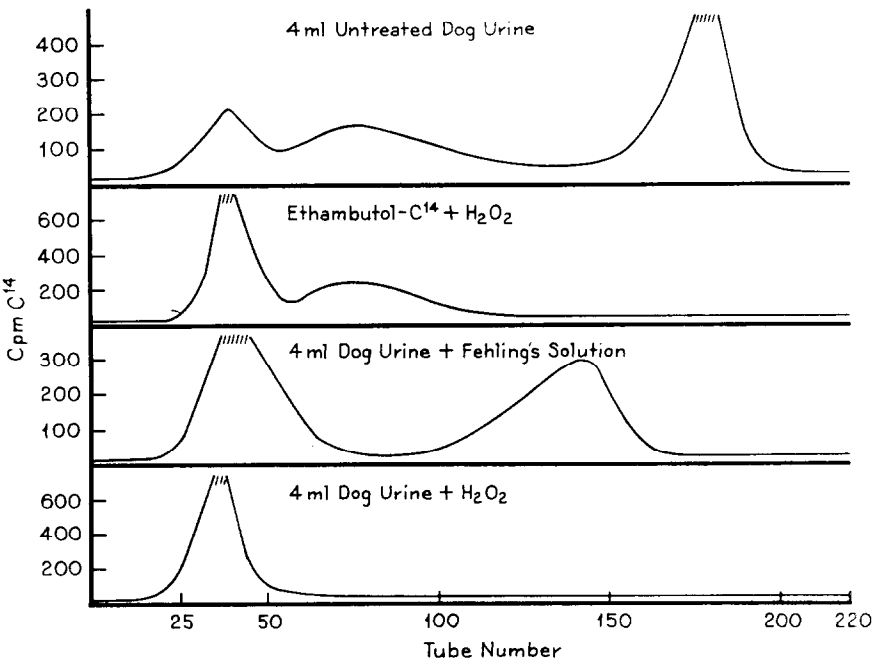


FIG. 7. Countercurrent distribution of dog urine 4–8 hr after ethambutol administration. Solvent system, *n*-butanol:ammonium hydroxide:water (4:1:5); 220 transfers.

Metabolite II and ethambutol-^{14}C in the urine, upon treatment with peroxide, were both oxidized to the dicarboxylic acid, as was demonstrated by their disappearance from the urine and the concurrent increase in the amount of the acid metabolite I. When subjected to milder oxidation with Fehling's solution, the material of intermediate polarity in the urine was again oxidized to the dicarboxylic acid, while the ethambutol-^{14}C remained unaltered. Also, the oxidation of ethambutol by peroxide resulted in the production of two materials having distribution coefficients similar to those of the dicarboxylic and aldehyde derivatives (Fig. 7). Chromatographic analysis of these substances demonstrated that they had mobilities which were the same as those of the two metabolites in similar systems and further indicated that these two oxidation products of ethambutol were the same as those arising from the *in-vivo* oxidation of the drug in the dog. The Rs's of these oxidation products (i.e. chromatographic mobility as compared with authentic 2,2′-ethylenediimino butyric acid and to the aldehyde metabolite II of urine) are presented in Table 6.

Since it was clear that the metabolism of ethambutol (and its L-isomer) involved its oxidation to the corresponding acid via the aldehyde intermediate, it became of interest to determine whether the drug was a substrate for mammalian or yeast-purified alcohol dehydrogenase. Under conditions of incubation *in vitro*, both ethambutol and its L-isomer acted as substrates for the liver alcohol dehydrogenase, as was

TABLE 6. PAPER CHROMATOGRAPY OF H_2O_2 OXIDATION PRODUCTS OF ETHAMBUTOL-^{14}C

Material	Rs system I*	Rs system II	Rs system III
Oxidation product 1	1·0	0·98	1·1
Oxidation product 2		0·98	1·0

* I *n*-Butanol:ammonium hydroxide:water (4:1:5).
II Isopropanol:ammonium hydroxide:water (4:1:5).
III Isopropanol:acetic acid:water (4:1:5).

indicated by the production of reduced DPN, but were poorer substrates for the enzyme than was ethanol (Fig. 8). The result of this enzymatic reaction was the aldehyde oxidation product. No significant differences existed in rates of oxidation of the two isomers. Yeast enzyme could also oxidize these drugs but acted at a much slower rate than the liver enzyme.

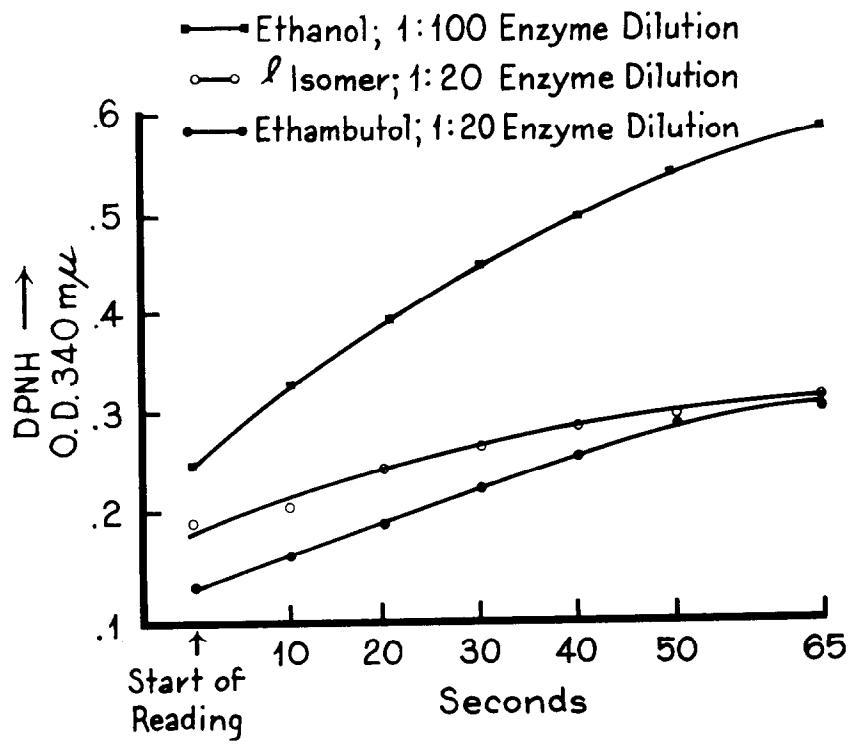


FIG. 8.The *in-vitro* oxidation of ethambutol and the L-isomer by liver alcohol dehydrogenase.

By the use of countercurrent distribution it was possible to isolate small amounts of the aldehyde resulting from the *in-vitro* oxidation of ethambutol by horse liver alcohol dehydrogenase (Fig. 9). The results indicated that this material had a distribution coefficient similar to that of the intermediate material in dog urine determined to be aldehydic in nature.

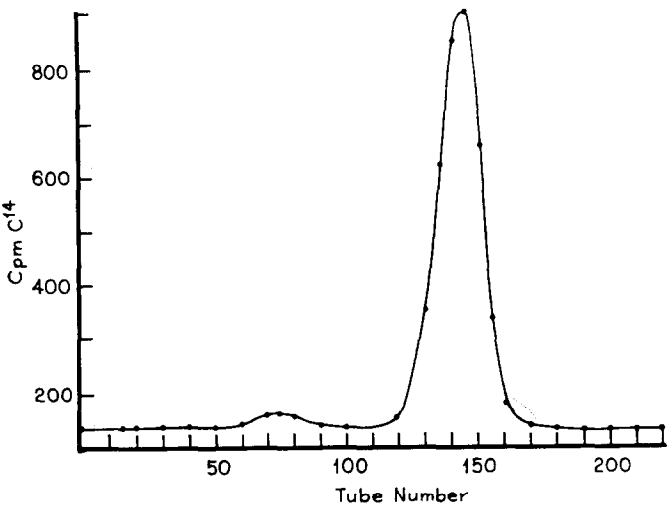


FIG. 9. Countercurrent distribution of ethambutol-^{14}C incubated 48 hr at 37° with liver alcohol dehydrogenase. Solvent system, *n*-butanol:ammonium hydroxide:water (4:1:5); 220 transfers.

Whole rat liver homogenate and the enzymes present in both the soluble and microsomal fractions of rat liver were also capable of metabolizing ethambutol to a single product, characteristically more polar than the drug. Countercurrent distribution analysis and paper chromatography indicated that this product was the initial aldehyde oxidation product of ethambutol. These data are presented in Fig. 10.

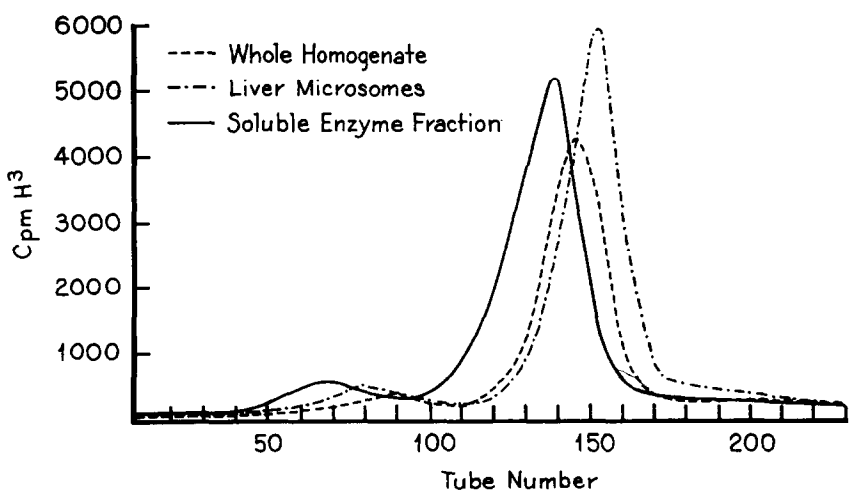


FIG. 10. Countercurrent distribution of ethambutol-^{3}H incubated with whole homogenate, soluble enzymes, and microsomes of rat liver. Solvent system, *n*-butanol:ammonium hydroxide:water (4:1:5), 220 transfers.

Oxidation of this material with hydrogen peroxide resulted in a radiolabeled product with a countercurrent mobility similar to that of the acid derivative of ethambutol and provided further evidence for the view that this material was aldehydic in nature and the same as that observed *in vivo*.

The soluble liver enzymes present in the supernatant fraction were more active in metabolizing ethambutol than were the tri-phosphopyridine-nucleotide-dependent enzymes of the liver microsomes. When incubated with 500 μg of ethambutol, soluble liver enzymes equivalent to 0·8 g of liver were able to transform 6% of the drug while microsomes equivalent to 3·2 g of liver were required to transform 5% of the parent compound.

On the basis of these data the main route of metabolism of the D- and L-isomers of 2,2′-(ethylenediimino)-di-1-butanol appeared to be an initial oxidation of the alcohol to an intermediate, aldehydic in nature, and the conversion of this aldehyde to the corresponding dicarboxylic acid, 2,2′-(ethylenediimino)-di-butyric acid. A scheme for the metabolism of ethambutol (or the L-isomer) is presented in Fig. 11.

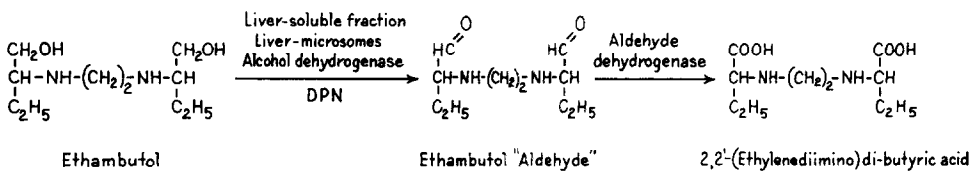


FIG. 11. Metabolic transformations of ethambutol.

DISCUSSION

The results reported herein point out major differences in the distribution and metabolism of ethambutol and its L-isomer and offer an example of stereochemical specificity. The influence of stereochemical configuration upon the pharmacological disposition of a drug is not unique to ethambutol; differences in the pharmacology and metabolism of the isomers of Nirvanol, Mesantoin, Arterenol, and Doriden have been reported.[14–16] The importance of stereochemical configuration upon toxicity has also been reported; L-penicillamine is toxic in rats, while the D-isomer is devoid of toxic manifestations.[17] Similarly the toxicity of 2,2′-(ethylenediimino)-di-1-butanol in rats, under conditions of chronic administration, is related to configuration, the *l*-isomer being more toxic than ethambutol (Moser, unpublished data).

Differences in the excretion of ethambutol-^{14}C and the ^{14}C-L-isomer have been noted, but no adequate explanation of this finding is at present available. Possibly, metabolic products of the L-isomer, or the parent compound itself, may become more firmly attached to some body structure than is ethambutol or its transformation products, thus resulting in a slower, less than quantitative excretion of the former from the body. The similar affinity of both isomers for erythrocytes suggests that this tissue is not responsible for the preferential retention of the L-isomer.

The differences in excretion can also explain the quantitatively greater oxidation of the L-isomer, since the longer the compound remains in the circulation the greater would be its enzymatic transformation. This hypothesis is supported in part by the observation that the alcohol dehydrogenase enzyme has a similar affinity for either

isomer, neither ethambutol nor the L-isomer being oxidized at significantly different rates.

The findings that the soluble enzymes of rat liver are capable of the metabolism *in vitro* of ethambutol, and that the drug is a substrate for purified alcohol dehydrogenase, are consistent with the nature of the metabolites produced *in vivo*. The initial metabolite is an aldehydic material resulting most probably from the action of an alcohol dehydrogenase enzyme, and the second the corresponding acid resulting from the action of an aldehyde dehydrogenase. Enzymes of this type are located in the soluble portions of the cell. The metabolism of ethambutol by the microsomal enzymes is well within the capacity of these enzymes. The relative quantitative importance of the microsomal as compared with the soluble fraction (presumably containing alcohol dehydrogenase) for conversion of ethambutol to the oxidation product in the intact animal, however, cannot be ascertained by the experiments described.

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