Freeze-Drying of Oxyhemoglobin: Protection Against Oxidation in the Presence of EDTA Salts, Sulfonic Acid Buffers, and Pantothenic Acid Derivatives

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Abstract □ Hemoglobin was freeze-dried in the presence of salts of EDTA, sulfonic acids used as buffers, or derivatives of pantothenic acid. At 0.25 M most of the compounds effectively inhibited the formation of methemoglobin. The various model compounds used (sodium zinc EDTA, tris(hydroxymethyl)methylaminopropanesulfonic acid, and DL-pantothenol) produced similar decreases in methemoglobin formation as a function of the concentration of protective agent between 0.01 and 0.20 M. Experiments on the storage of the freeze-dried materials revealed substantial denaturation of oxyhemoglobin after 12 months at 4°C. On the whole, these compounds were less effective than amino acid salts, during both desiccation and storage. The multiplicity of compounds that inhibit the denaturation of hemoglobin during freeze-drying indicates that their mode of action is nonspecific.

Keyphrases □ Oxyhemoglobin—oxidation to methemoglobin during freeze-drying, protection by EDTA salts, sulfonic acid buffers, and pantothenic acid derivatives □ EDTA salts—protection of oxyhemoglobin during freeze-drying, prevention of methemoglobin formation □ Sulfonic acid buffers—protection of oxyhemoglobin during freeze-drying, prevention of methemoglobin formation □ Pantothenic acid derivatives—protection of oxyhemoglobin during freeze-drying, prevention of methemoglobin formation

Oxyhemoglobin has been freeze-dried under various conditions, and it has been definitely established that desiccation leads to oxidation of the heme iron and to the formation of methemoglobin (metHb) representing up to 60% of the original hemoglobin (1-3). Until now, the only way of obtaining nondenatured freeze-dried hemoglobin has been to add a sufficient concentration of a protective agent before freezing the solution. The demonstration by Smith and Pennell in 1952 (4) of a protective effect of glucose has been followed more recently by exploration of a series of chemical compounds such as amine buffers (5), carbohydrates (6), and macromolecules (7), for which dose-response relationships have been established. Better understanding of protective agents would be useful in stabilizing solutions of hemoglobin for eventual use as a blood substitute and in other biotechnologies.

We describe here experiments with representatives of three series of chemical compounds not previously studied as protective agents in freeze-drying, chosen for the following reasons: EDTA salts, because of their effects on the process of oxidation of hemoglobin (8, 9); buffer compounds, to follow up our earlier studies of Tris derivatives (5); and derivatives of pantothenic acid, because their parent molecule is structurally related to some protective compounds already known—β-alanine and quaternary carbon polyols (2, 5).

Table I—Physiological Properties of Hemoglobin after Freeze-Drying in the Presence of 250 mM of Different EDTA Salts

EDTA Salt	*n*	Oxyhemoglobin, %	Methemoglobin, %	pH	Hill coefficient
Dipotassium	3	85.3	14.0	5.31	1.96
Sodium zinc	3	91.9	6.5	7.07	2.18
Dipotassium magnesium	3	89.7	3.5	7.30	2.36
Disodium magnesium	3	92.8	2.9	7.26	2.52
Tetrasodium	3	84.4	1.5	8.56	2.88
Tripotassium	3	86.9	2.8	7.70	2.52

EXPERIMENTAL

Reagents—A solution of human hemoglobin was prepared from citrated blood samples taken 3 weeks earlier. The red blood cells were separated and washed in 0.15 M NaCl, and then hemolyzed in deionized water. The stromata were separated by centrifugation twice at 25,000×*g* for 30 min and decantation of the hemoglobin solution. This solution was dialyzed against deionized water at 4°C for 15 h, then centrifuged once again to remove the remaining stromata and adjusted to a hemoglobin concentration of 100 g/L. The EDTA salts used were: dipotassium·2 $_2O$[1], sodium zinc·4H_2O[2], dipotassium magnesium[1], disodium magnesium·H_2O[2], tetrasodium[1], and tripotassium[3]. The pantothenic acid (vitamin B_5) derivatives were: calcium D-pantothenate[4], DL-pantothenic alcohol[4], D-pantothenic alcohol[4], (−)-D-pantoyllactone[4], and sodium DL-pantoyltaurinate[4]. The buffers were 3-(*N*-morpholino)propanesulfonic acid (I)[4], *N*-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (II)[4], 2[(2-amino 2-oxoethyl)amino]ethanesulfonic acid (III)[2], *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (IV)[2], *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (V)[2], and tris(hydroxymethyl)methylaminopropanesulfonic acid (VI)[2].

Procedures—In the initial series of experiments, the samples to be freeze-dried were made up of a homogeneous mixture of 5 mL of an aqueous 100-g/L solution of hemoglobin and 5 mL of an aqueous 0.5 M solution of the protective agent being studied. This made it possible to verify that these materials were indeed soluble. Next, for one agent from each of the three chemical series we looked at the dose-response relationship, plotting the percentage of methemoglobin formed *versus* the molar concentration of the protective agent, from 0.01 to 0.2 M, at a constant hemoglobin concentration as before and with a volume of 10 mL before freeze-drying. Each sample was studied in triplicate.

More detailed studies were carried out with EDTA. First, a series of excellent freeze-dried materials, obtained from 10 mL of solutions containing 100 g/L of hemoglobin and 0.25 M of each of its salts, were stored in darkness for 12 months in air at 4°C. Next, samples freeze-dried in the presence of 0.01-0.20 M sodium zinc EDTA were stored in the same conditions for 12 months. Finally, we wished to find out (as has not yet to our knowledge been systematically studied) whether the concentration of hemoglobin influenced the proportion of methemoglobin formed, at a constant concentration of protective agent chosen to give a level of oxidation that was neither zero nor too high. The experiments were carried out on 10 mL of freeze-dried material, with 0.05 M sodium zinc EDTA and 250-1000 mg of hemoglobin.

The freeze-drying was carried out in a freeze-drying apparatus[5] under the following conditions. Samples (10 mL) were frozen at −40°C in the bottom of 100-mL flasks in the apparatus, followed by desiccation with the control set at 70% (heating of shelf 70% of time) and final temperature displayed as 4°C. The vacuum was broken and the freeze-dried materials were immediately stoppered in air. The samples were macroscopically examined (for appearance and color) and dissolved in 10 mL of Sörensen's 0.066 M phosphate buffer of pH 6.4, 7.4, or 7.8, depending on the pH before freeze-drying. The other examinations carried out were:

1. Estimation of methemoglobin by the method of Evelyn and Malloy (10).

[1] Prolabo.
[2] Fluka.
[3] Touzart-Matignon.
[4] Sigma.
[5] USIFROID SMH 15; Maurepas, France.

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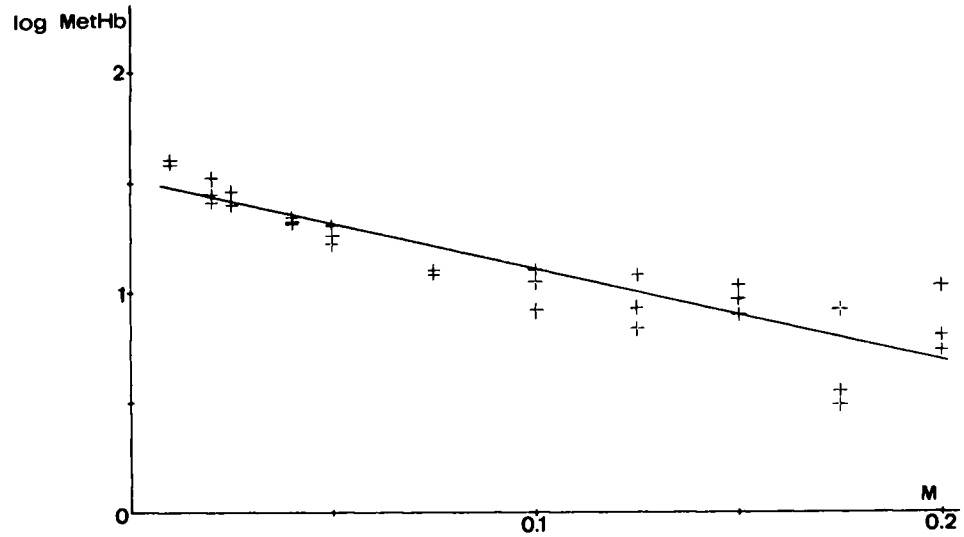

Figure 1—*Linear regression between the log methemoglobin (%) and molar concentration (0.01 to 0.20 M) of Na-Zn EDTA.*

2. Measurement of oxyhemoglobin in the aqueous solution with an oximeter[6], without correction for the concentrations of methemoglobin.

3. Determination of the pH[7] of the reconstituted hemoglobin solution.

4. Plotting of the dissociation curve[8] of the hemoglobin at 37°C and $P\,CO_2$ of 40 Torr, and calculation of the Hill coefficient.

5. Plotting of the spectrum[9] of the hemoglobin from 500 to 700 nm.

6. Calculation of the concentration-protection relationships using a programmable calculator[10] linked to a plotter.

RESULTS AND DISCUSSION

In all our previous experiments on freeze-drying carried out under similar conditions, unprotected hemoglobin contained 49 ± 8% of methemoglobin (metHb) after freeze-drying (n = 45). Its oxygen saturation was 56 ± 5% (n = 29), and its cooperativity was lost (Hill coefficient = 1.76 ± 0.07, compared with a normal value of >2.6).

Protection Due to EDTA Salts—On coming out of the freeze-drier, the samples were red and powdery; they dissolved rapidly and totally. All the EDTA salts studied here, except for dipotassium EDTA, seemed to be protective agents that inhibited the formation of methemoglobin, giving <10% methemoglobin (Table I). The tetrasodium salt was the most effective, but it led to the lowest saturation values and a pH very different from physiologic values. Least-squares best estimates were calculated for straight lines on Hill plots; the Hill coefficients obtained demonstrated that the physiological properties of the hemoglobin were well preserved, except with the dipotassium salt.

We arbitrarily chose the sodium zinc salt for the other studies. The color of the dry freeze-dried materials, ranging from brown to red, indicated decreasing proportions of methemoglobin as the concentration of protective agent increased. The samples showed no turbidity when reconstituted with a pH 7.4 buffer, but the more altered the hemoglobin, the slower they were to dissolve. The relationship between the percentage of methemoglobin (y-axis) and the percentage of oxyhemoglobin (x-axis) fitted the equation $y = -x + 97.1$ (n = 33, $r = -0.97$, $p < 0.001$). There was a linear relationship between the logarithm of the proportion of methemoglobin and the molar concentration of protective agent: $y = -4.12x + 1.51$ (n = 33, $r = -0.903$, $p < 0.001$) (Fig. 1). After 12 months, the freeze-dried materials obtained with the EDTA salts showed methemoglobin levels between 8.8% (with the tetrasodium salt) and 64.7% (with the dipotassium salt). The order of effectiveness of the various salts was the same as at time zero. The saturations were low (56–70%), especially in the presence of tetrasodium EDTA, although the spectrum of the hemoglobin of this sample was altered very little.

The samples containing various concentrations of sodium zinc EDTA displayed proportions of methemoglobin between 50.9 and 26.8%, yielding the correlation line $y = -185.6x + 58.9$ (n = 10, $r = -0.918$, $p < 0.001$); this line cuts the x-axis at 0.32 M, which is the hypothetical concentration of EDTA in a hemoglobin solution that will prevent any oxidation to methemoglobin during both desiccation and storage. This concentration is much higher than those we found with other protective agents under similar conditions (11), which indicates that EDTA salts are much less effective. The proportion of methemoglobin varied greatly with the concentration of hemoglobin, as seen in Fig. 2 ($y = 0.026x + 2.89$; n = 14, $r = 0.98$, $p < 0.001$). This relationship between hemoglobin and protective agent is in accordance with the previous relationship (Fig. 1): both show that hemoglobin was correctly protected only in certain narrowly defined conditions of concentration. This sensitivity of hemoglobin to small changes in concentration was found also for the other classic protective agents (such as glucose or sucrose) for which we have established equations of the same type[11].

Table II—Physiological Properties of Hemoglobin after Freeze-Drying in the Presence of 250 mM of Buffer Compounds

Buffer	n	Oxyhemoglobin, %	Methemoglobin, %	pH	Hill coefficient
I	3	93.6	2.2	6.15	2.90
II	3	99.0	1.9	6.65	2.40
III	3	65.5	47.1	6.35	—
IV	3	94.6	4.6	6.23	2.01
V	3	96.0	2.2	6.33	2.07
VI	3	97.6	3.5	7.13	3.00

Protection Due to Buffer Compounds—Buffer III is poorly soluble in aqueous media and had no protective effect, whereas the five other buffers gave satisfactory protection (Table II). The structure of these compounds (sulfonic acids) and their concentrations produced acid solutions despite their being dissolved in an alkaline buffer solution. At these pH values, the Barcroft curve was displaced towards low affinities and the $p50$ values were high (21–45 Torr).

The dose-response relationship was studied with VI. At concentrations of ≥0.075 M it gave satisfactory levels of methemoglobin, *i.e.* <10%. The relationship between the logarithm of the percentage of methemoglobin and the concentration of VI took the form $y = -5.76x + 1.52$ (n = 33, $r = -0.867$, $p < 0.001$) (Fig. 3). Here too, the correlation between methemoglobin and oxyhemoglobin was very significant: $y = -0.75x + 75.62$ (n = 33, $r = -0.975$, $p < 0.001$). Two samples of hemoglobin freeze-dried in the presence of VI or V were stored for 12 months at 4°C; the levels of oxyhemoglobin and methemoglobin were then found to be 76% and 22%, respectively, and their respective Hill coefficients were 2.13 and 1.66. As with the EDTA salts, these protective agents seemed to be greatly inferior to the amino acid salts and sucrose studied previously (11).

Protection Due to Derivatives of Pantothenic Acid—These derivatives were quite difficult to study, because D-pantothenol is very viscous and dissolves poorly in water, while (−)-D-pantoyllactone yielded a poorly soluble freeze-dried material, whose solution was slightly turbid, brown, and in need of centrifugation. Calcium pantothenate yielded even greater turbidity, which prevented any precise estimation of methemoglobin in the centrifugation supernatant. Nevertheless these compounds were effective protective agents, as seen in Table III. The correlations established above were again found for log (methemoglobin percentage) *versus* molarity of the protective agent

[6] Radiometer OSM 2 Hemoximeter.
[7] Corning 161 Blood-gas analyzer.
[8] Hem-O-Scan analyzer Aminco.
[9] Shimadzu spectrophotometer.
[10] Hewlett-Packard Calculor 9810 A.
[11] Unpublished results.

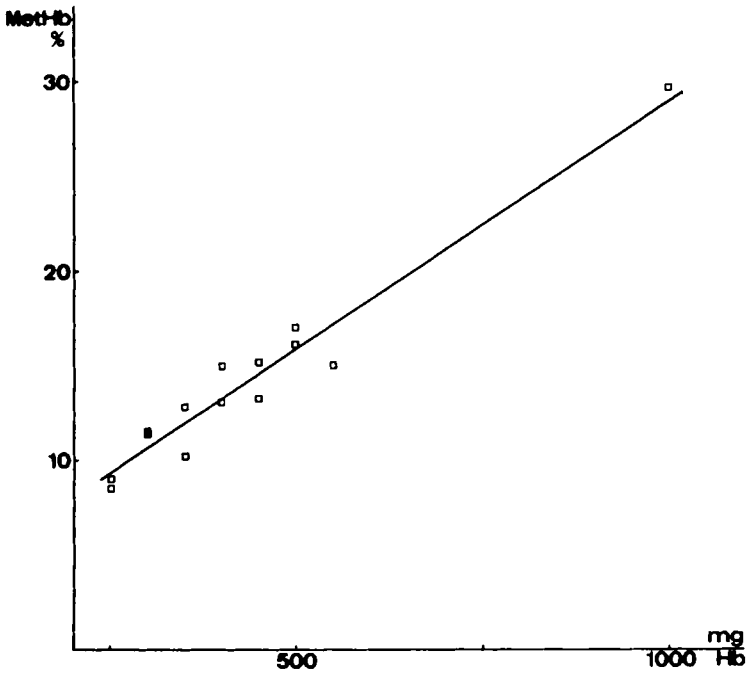

Figure 2—*Linear regression between methemoglobin (%) and the amount (or concentration) of hemoglobin during freeze-drying with 50 mM Na-Zn EDTA.*

DL-pantothenol [$y = -7.19x + 1.64$ (Fig. 4) ($n = 33, r = -0.95, p < 0.001$)] and for methemoglobin *versus* oxyhemoglobin [$y = -0.86x + 84.6$ ($n = 33$, $r = -0.98, p < 0.001$)].

CONCLUSIONS

This study has demonstrated the protective effect during the freeze-drying of hemoglobin of representatives of three different chemical series, adding to those already known (5–7). With EDTA salts, where only the cation and the pH differed, the proportion of oxyhemoglobin was generally low, and pH seemed to be involved, since the extreme pH values gave both the best and the worst results. With one exception, the sulfonic acids used as biological buffers were effective protective agents. The same was true for the pantothenate family that we have investigated, since they derive from both β-alanine and a quaternary carbon alcohol. This remark suggests that there may be possible structure-activity relationships in this series.

On the other hand, three reasons lead us instead to conclude that a nonspecific antioxidant action is present: (*a*) the multiplicity and the variety of chemical families in which protective agents for hemoglobin have already been found—carbohydrates, amine buffers, macromolecules, amino acids, *etc.*, (*b*) the observation by ESR that glucose, β-alanine, or Tris, taken as models, do not interact with heme iron and its environment (12), and (*c*) the result with the same models using circular dichroism (CD) that the absence of protector only weakly influences the conformation in the vicinity of the heme and alters the secondary structure by slightly increasing the helicity. The CD spectra also suggest that the protector may not enter into the heme pocket, since one would then observe much greater spectral changes in the visible region and the Soret band (13).

Table III—Physiological Properties of Hemoglobin after Freeze-Drying in the Presence of 250 mM of Pantothenic Acid Derivatives[a]

Derivative	*n*	Oxyhemoglobin, %	Methemoglobin, %	pH	Hill's Number
D-Pantothenic acid, hemicalcium salt	3	97.4	—	5.61	—
DL-Panthenol	3	99.3	2.3	7.29	2.51
D-panthenol	3	98.3	3.4	7.35	2.49
(−)-D-Pantoyllactone	3	75.3	23.3	7.28	2.11
DL-Pantoyltaurine sodium salt	3	100.0	2.0	8.28	2.53

[a] Derivatives were dissolved in buffer, pH 6.38.

Taking in account the preceding remarks, it appears that the oxidation of ferrous iron remains the main denaturer of hemoglobin and that the estimation of methemoglobin is the first and one of the best tests to evaluate the effects of freeze-drying on hemoglobin. The mechanism of action of the protectors is still poorly understood, but our current experiments link this action with concomitant effects of vacuum and removal of water. It is not impossible that

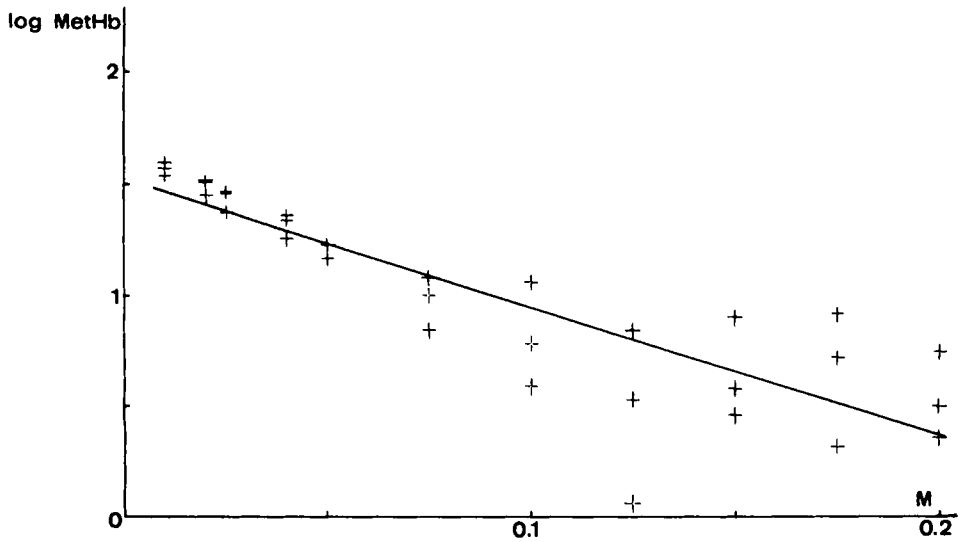

Figure 3—*Linear regression between the log of methemoglobin (%) and molar concentration (0.01 to 0.20 M) of VI.*

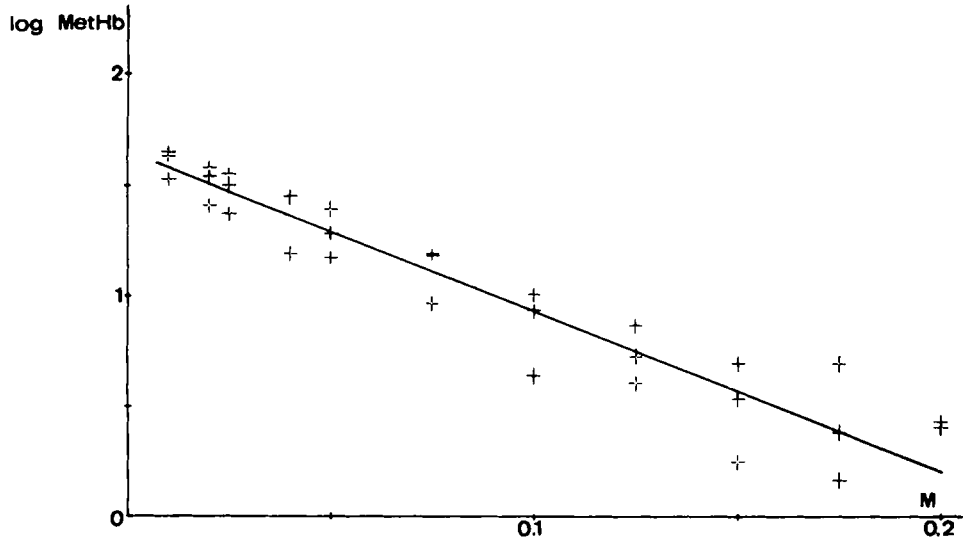

Figure 4—*Linear regression between the log of methemoglobin (%) and molar concentration (0.01 to 0.20 M) of* DL-*panthenol.*

such protectors "modify" the structure of globin as has been described for some proteins and glycerol by Gekko and Timasheff (14).

The changes in the proportion of methemoglobin as a function of concentration of the protective agent always fitted a hyperbola that could be linearized by a logarithmic transformation, giving a straight line with a negative slope. The three correlation lines thus obtained had slopes of the same order of magnitude, which indicates that these three model compounds are fairly similar in effectiveness. However, they are much less effective, during both freeze-drying and storage, than the amino acid salts that we have studied previously. This difference in behavior, which undoubtedly derives from quite specific physicochemical properties of the protective agents, remains unexplained.

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Disposition Kinetics of Ethambutol in Nephrectomized Dogs

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Abstract □ The effect of nephrectomy on the disposition of ethambutol was investigated in seven adult mongrel dogs: five were nephrectomized and two served as the control. Each dog was intravenously administerd 500 mg ethambutol, followed by blood sample collection for 12 h. Total urine was collected over 24 h from the normal control dogs. Ethambutol contents in plasma and urine were assayed by a GC method. The nephrectomized group and the control group exhibited differences in the following pharmacokinetic parameters: half-life, 5.0 *versus* 4.1 h (significant at $p < 0.1$); total body clearance, 8.4 *versus* 13.2 mL/min/kg (significant at $p < 0.1$); and volume of distribution, 2.7 *versus* 3.8 L/kg (significant at $p < 0.1$). Comparison of pharamacokinetic parameters among rabbits, dogs, and humans revealed distinct interspecies differences with regard to total body clearance, renal clearance, volume of distribution, and fractional renal excretion. One comparable parameter shared by all species is the β-phase half-life.

Keyphrases □ Ethambutol—disposition, pharmacokinetics in the dog, effect of nephrectomy, interspecies comparison □ Pharmacokinetics—ethambutol disposition in the dog, effect of nephrectomy, interspecies comparison □ Distribution—ethambutol in the dog, effect of nephrectomy, pharmacokinetics, interspecies comparison

Ethambutol (I), an antitubercular agent, is used alone or in combination with other drugs for the treatment of tuberculosis. The absorption and excretion of I has been studied in rats and mice (1), dogs (2, 3), and human subjects (4–6). In mice receiving single oral doses of I, peak serum concentrations were reached within 1 h of administration, and the compound

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