# TOXICOLOGICAL EVALUATION OF ASBESTOS SUBSTITUTE

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## INTRODUCTION

Epidemiological and experimental studies have proved that asbestos induces certain pathological changes in the lung like fibrosis knows as asbestosis and two forms of malignancies, i.e. mesothelioma and bronchogenic carcinoma.[30,35] In order to safeguard the workers from the hazardous effects of these fibres, scientists all over the world are trying to replace them with other natural and man-made mineral fibres. Among them, wollastonite is a promising asbestos substitute under trial in India. They are acicular or fibrous calcium silicates ($CaSiO_3$), and have attained additional significance due to their high thermal resistance properties. The use of wollastonite in ceramic tiles, etc., has given this mineral considerable attention as a substitute for asbestos fibres.[1,25] A few reports are available on their biological effects. Skaug and Gylseth[38] have reported, on the basis of hemolytic studies of two natural and three synthetic calcium silicates, that synthetic silicates are more toxic than the natural. Bolton et al[2] have reported no major pulmonary damage in rats exposed to three different varieties of calcium silicate insulation materials. Moreover, a few cases of lung fibrosis, pleural thickening, chronic bronchitis and impairment of lung ventilatory capacity in wollastonite exposed workers have also been reported.[11,20] However, no report is available on the biological activity of Indian varieties of wollastonite dusts. Therefore, in the present study, three varieties of Indian wollastonite, namely, kemolit A-60, kemolit-N and kemolit ASB-3 have been evaluated for their toxicity. Besides cytotoxic studies *in vitro* and fibrogenic responses *in vivo*, the effect of these fibres on the pulmonary xenobiotic metabolizing enzyme system was also evaluated, to monitor their influence in the presence of other carcinogens, if present in the system simultaneously either by smoking or from the other environmental sources. The results obtained from these studies were compared with chrysotile, the most toxic variety of asbestos and also a carcinogenic enhancer in the presence of tobacco smoke.[3]

## MATERIALS AND METHODS

### Dust

Wollastonite dust samples, kemolit A-60, kemolit-N and kemolit ASB-3 were obtained from Mr. Salil Singhal, Director, Wolkem Private Ltd. Udaipur (India). Particle size below 30 u were prepared as described by Zaidi.[43] Chrysotile UICC standard reference sample particle size $<30\ \mu$ was obtained as a gift from Dr. J.B. Leinweber, Johns-Manville, U.S.A.

### Chemicals

Benzo(a)pyrene, 3-hydroxy benzo(a)pyrene, styrene epoxide, 1-chloro, 2,4,-dinitrobenzene and bovine serum albumin were procured from Sigma Chemical Company, USA. All the other chemicals and reagents were either purchased from V.P. Chest Institute, New Delhi, India or Sisco Research Laboratory (SRL) Bombay, India, and were of analytical grade.

### Hemolytic Studies

The lysis of 0.2% suspension of human erythrocyte in 0.01M Tris-HCl buffer pH 7.35 in 0.15M NaCl caused by 2 mg/ml each of different dusts was measured at 37°C after two hours except chrysotile where it was 10 minutes to avoid adsorption.[33]

### Treatment of Animals

Female albino rats from ITRC Colony, weighing 150-180 gm, were used. The dried dusts and 0.15 M NaCl were separately autoclaved at 15 lbs pressure for 15 min. The dusts were separately suspended in 0.15 M NaCl just before inoculation. The animals were divided into five groups. Intratracheal treatment of animals with dust were done according to the procedure as described by Zaidi.[43]

Each animal of the experimental groups was instilled intratracheally with 5 mg of different dust samples separately, suspended in 0.5 ml of normal saline. Control groups received 0.5 ml of normal saline solution only. The animals were maintained on commercial pellet diet, supplied by Hindustan Lever Limited, Bombay, India, and tap water *ad libitum*. The animals were sacrificed at 90 days after the instillation of dusts. Lungs were taken out, weighed and a portion was fixed in 10% formal saline for histopathological studies, while the other portion was cut into small pieces and dried at 110°C for chemical estimation. Another set was taken for microsomal and cytosolic fractionations.

### Histopathological Studies

Representative 5 $\mu$ paraffin sections were cut and stained with hematoxylin-eosin and VanGieson.

### Isolation of Microsomes

The rat lung microsomal fraction was isolated by the modified procedure of Johannesen et al.[22]

### Enzyme Assays

Benzo(a)pyrene hydroxylase was assayed by the fluorimetric technique as described by Dehnen et al.[8] The quantitation of phenolic metabolite was based on comparison of fluorescence to a standard solution of 3-hydroxy benzo(a)pyrene.

Epoxide hydratase activity was assayed by the fluorimetric technique, according to the method of Dansette et al.[6] by using styrene epoxide as substrate.

Glutathione-S-transferase activity was determined by the procedure, described by Habig et al.,[16] by using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate.

### Chemical Estimation

Microsomal cytochrome P-450 was quantitated from carbon monoxide plus dithionite reduced difference spectra as described by Omura and Sato.[31] An extinction coefficient of 91,000 $cm^{-1}M^{-1}$ was used for absorbance change between 450 and 490 nm.

Glutathione content was measured in rat lung cytosolic fraction according to the method of Ellmann.[12]

Ascorbic acid content was estimated in lung cytosol according to the procedure of Schaffert and Kingsley.[34]

Enzymatic and non-enzymatic lipid peroxidation was determined by the procedure of Ottolenghi[32] as modified by Hunter et al.[19] estimating the malonaldehyde formed with 2-thiobarbituric acid.

Hexosamine and sialic acid were estimated in the fresh tissue by the methods of Dische and Broenfreund[10] and Warren respectively. Uronic acid and collagen were estimated by the method of Dische,[9] and Stegmann et al.[41] respectively in dry tissue. Phospholipids were extracted from dry tissue in chloroform: methanol (2:1) and were estimated by their phosphorous content by the method of Fiske and Subba Row.[13]

Protein content in trichloroacetic acid precipitate was estimated by the method of Lowry et al.[27] by using crystalline bovine serum albumin as standard.

## RESULTS

### In vitro Studies

Table I shows that all the wollastonite dust samples induced hemolysis and the order of hemolysis was 43.4%, 41.2% and 35.3% by kemolit A-60, kemolit-N and kemolit ASB-3 respectively. In comparison to chrysotile all the samples were less hemolytic.

### In vivo Studies

*Fibrogenic Response.* The changes in the chemical composition and dry weight of the lung by different wollastonite samples are recorded in Table II. When compared with chrysotile,[7] the increase in the collagen content and phospholipids by kemolit A-60 was significantly very high. There was no significant increase in the mucopolysaccharides by these dusts. Kemolit A-60 increased only the content of sialic acid.

The histopathological studies revealed mild to moderate amount of fibrosis of the lung alveoli (Figure 1). The animals exposed to Kemolit A-60 showed peribronchiolar areas of fibrosis (Figure 2) which was not found with any other dust. In some cases collection of chronic inflammatory cells and few abscesses were also seen on scanning the tissue (Figures 3 and 4).

Table I

Comparative Hemolysis of Chrysotile and Wollastonite Using Rat Erythrocytes

| Dust sample | % Hemolysis |
|---|---|
| Chrysotile | 72.16±3.29 |
| Kemolit A-60 | 43.37±2.50 |
| Kemolit - N | 41.19±3.03 |
| Kemolit ASB-3 | 35.28±1.65 |

The values represent mean of six separate experiments ± S.D.

### Lung Weight

A significant increase in lung weight of all the experimental animals was observed, kemolit A-60 showed a higher increase in the lung weight as compared to kemolit-N and kemolit ASB-3 (Figure 5).

### Effects of Different Dusts on Lung Microsomal and Cytosolic Fractions

Figure 6 shows the increase in the cytochrome-P-450 content by different dusts. Chrysotile showed the maximum increase followed by kemolit A-60. Activities of benzo(a)pyrene hydroxylase and epoxide hydratase is recorded in Figure 7 and 8. Among all the dust samples kemolit A-60 induced maximum increase but in comparison to chrysotile the increase was of lower magnitude. The alteration in the activity of glutathione-S-transferase is recorded in Figure 9. Chrysotile decreased the activity of this enzyme significantly, while kemolit A-60 decreased the activity 9% which was three times less than observed by chrysotile. Kemolit-N and kemolit ASB-3 increased the activity of glutathione-S-transferase.

### Effect on Water Soluble Antioxidants

Statistically, chrysotile and kemolit A-60 induced significant decrease in the content of ascorbic acid as reported in Figure 10, while glutathione content was significantly decreased only by chrysotile (Figure 11).

### Effect on Microsomal Lipid Peroxidation

Chrysotile and kemolit A-60 induced significant lipid peroxidation both enzymatically and non-enzymatically, followed by kemolit-N. There was no change by kemolit ASB-3 (Table III).

Table II

Changes in the Lung Weight and Composition of Control and Wollastonite Treated Rats

| Parameters | Control | Kemolit A-60 | Kemolit N | Kemolit ASB-3 |
|---|---|---|---|---|
| Dry Weight (mg/g fresh tissue) | 204±23 | 237±21[d] | 218±19 | 205±19 |
| Lung protein (mg/g fresh weight) | 100±8.2 | 105±5.8[d] | 103±2.5 | 101.4±1.9 |
| Hexosamine (mg/100 mg fresh tissue) | 2.00±0.14 | 2.16±0.054 | 2.09±0.08 | 2.04±0.08 |
| Sialic acid (mg/100 mg fresh tissue) | 3.34±0.14 | 3.70±0.23 | 3.47±0.48 | 3.44±0.56 |
| Uronic acid (mg/g dry weight) | 23.38±3.98 | 28.10±4.84 | 26.65±3.57 | 25.96±2.71 |
| Collagen (mg/g dry weight) | 39.36±3.87 | 61.45±11.87[c] | 48.93±7.32 | 45.76±5.56 |
| Phospholipids (mg/g dry weight) | 7.96±0.46 | 13.31±0.33[a] | 9.31±0.09[b] | 8.57±0.13[d] |

The values are expressed as mean ± S.D. of six animals.

[a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.02$; [d] $p < 0.05$.

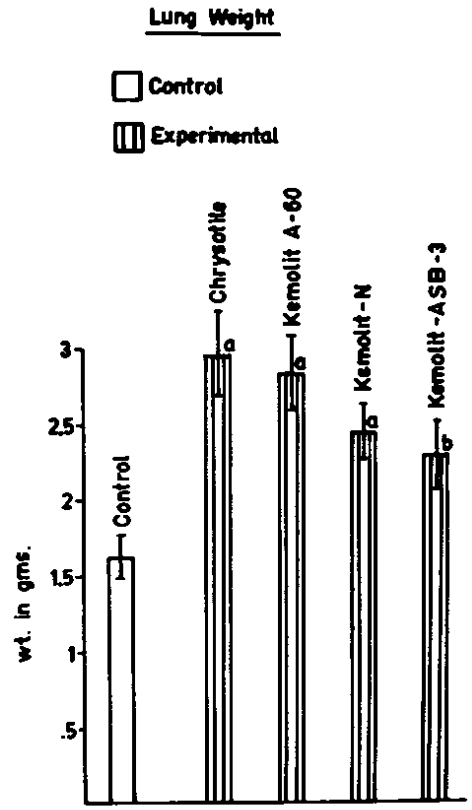


Figure 1. Section of rat lung tissue taken 90 days after the intratracheal inoculation of wollastonite showing interstitial fibrosis. 400×

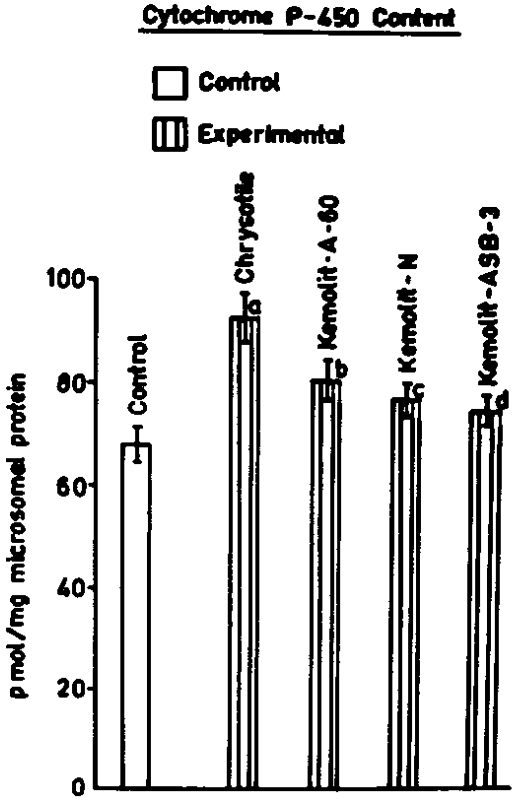


Figure 2. Section of rat lung tissue taken 90 days after the intratracheal inoculation of wollastonite showing peribronchiolar area of fibrosis. 400×

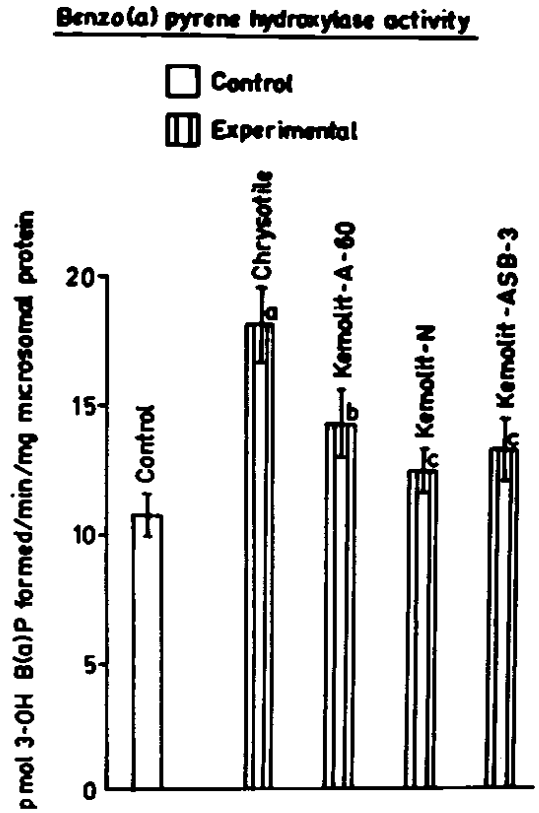


Figure 3. Section of rat lung tissue taken 90 days after the intratracheal inoculation of wollastonite showing inflammatory cells. 400×

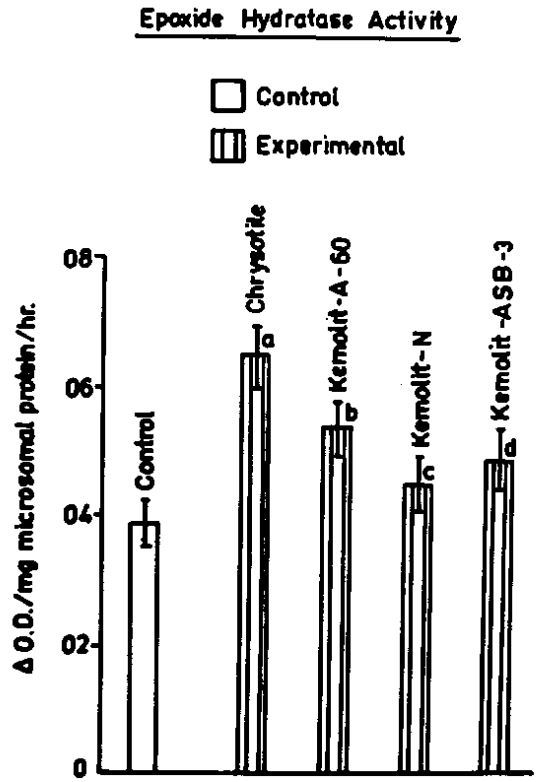


Figure 4. Section of rat lung tissue taken 90 days after the intratracheal inoculation of wollastonite showing abscess. 400×

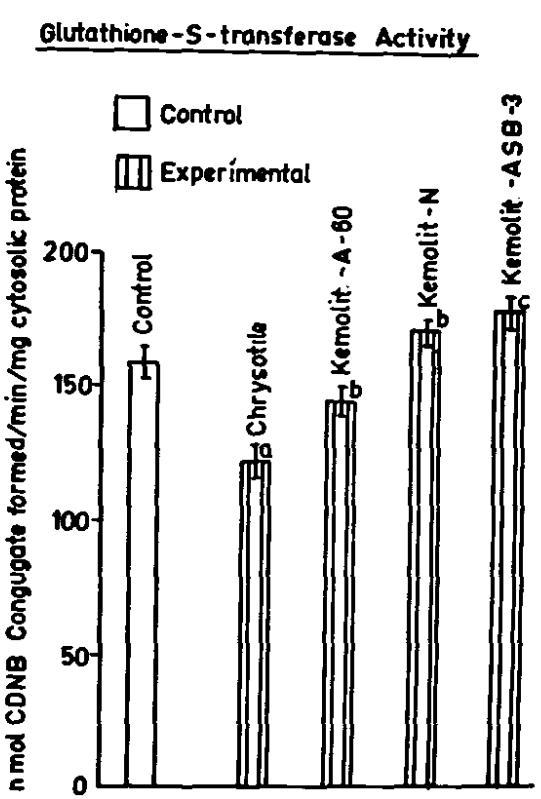


Figure 5. Fresh lung weight of control and dust treated animals. The values are expressed as mean ± SEM of six animals $^{a}p < 0.001$; $^{b}p < 0.02$.

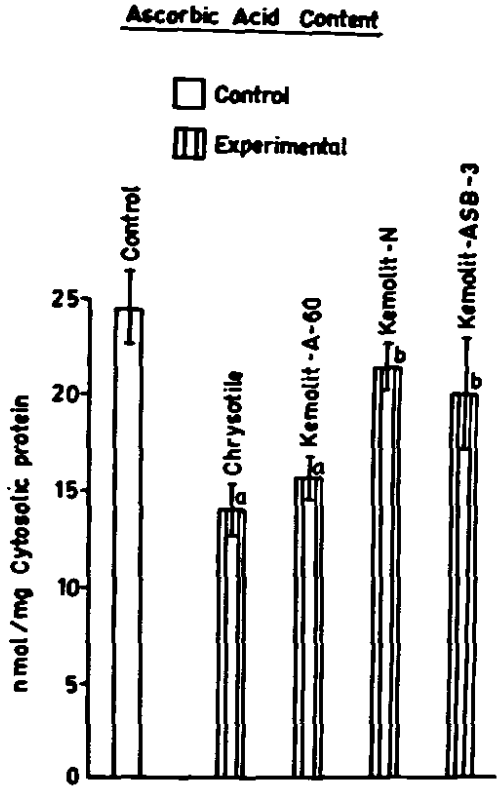


Figure 6. Lung cytochrome P-450 content of control and dust treated rats. Results represent mean ± SEM of six animals, $^{a}p < 0.001$, $^{b}p < 0.02$, $^{c}p < 0.05$, $^{d}p$ - N.S.

## DISCUSSION

In the present study among the three varieties of wollastonite, kemolit A-60 was found most toxic. The cytotoxic index of these samples were much less than chrysotile[18] using erythrocytes as *in vitro* model system[37] Kemolit A-60 was found to be the most fibrogenic dust. The fibrogenic pattern of these dusts tallied with those of Bolton et al.[2]

It appeared from the results that only kemolit A-60 could influence the activity of benzo(a)pyrene hydroxylase and epoxide hydratase in the diseased animals where fibrosis had already developed and collagen content was very high. However, when compared with chrysotile exposed animals where fibrosis had just begun and collagen content was lower than kemolit A-60 exposed animals, the activities of these enzymes were much less. Benzo(a) pyrene hydroxylase and epoxide hydratase play a crucial role in the formation of ultimate carcinogen derived from polynuclear aromatic hydrocarbons.[14,24] Further activation of these enzymes induced by chrysotile may aggravate the situation in the presence of other carcinogens, if present in the system. The effect of these dusts on glutathione-S-transferase activity was very interesting. This enzyme catalysed the conjugation of the ultimate carcinogens with glutathione in the lungs, which are eventually eliminated.[5] The inhibition of these enzymes and the reduction of glutathione by chrysotile decelerate the above reaction, hence providing the accumulated metabolites with the opportunity of interacting with DNA.[42] Similar results were also reported by Brown et al.[3] On the other hand kemolit ASB-3 increased the activity of glutathione-S-transferase significantly which could facilitate the elimination of reactive metabolites of the PAHs from the system. The other two dusts, i.e., kemolit A-60 and kemolit-N did not have any significant effect on the activity of this enzyme. Chrysotile inhibited the content of water soluble antioxidants, like glutathione and ascorbic acid significantly. It is important to note this, since antioxidants are known to inhibit tumors induced by PAHs.[23,36,39,40] Among the asbestos substitutes, kemolit A-60 decreased the content of ascorbic acid only and had no significant effect on glutathione but when compared with chrysotile the magnitude of decrease was much less. The decrease in ascorbic acid content by kemolit A-60 could be associated with its high fibrogenic response at this stage. Ascorbic acid is one of the important components of mammalian lungs defense against environmental pollutants.[26,28] It is closely associated with environmental stress in man and animals.[4] Therefore, its low level in lungs may hamper the defence of tissue against environmental pollutants. A higher rate of enzymatic and nonenzymatic lipid peroxidation was observed by chrysotile and kemolit A-60 while the induction

of LPO with kemolit-N was of lower magnitude and that with kemolit ASB-3 was insignificant. It is well documented that the induction of free radicals may be responsible for the pathogenicity produced by asbestos.[15,17,21,29]

From these studies it is evident that in comparison to chrysotile asbestos, wollastonites were less cytotoxic and did not bring significant alterations in the drug metabolizing enzyme system. Concluding this paper we would like to emphasize that kemolit-N lies at one end of the spectrum being closely followed by kemolit ASB-3 with kemolit A-60 as the most toxic form of wollastonite.

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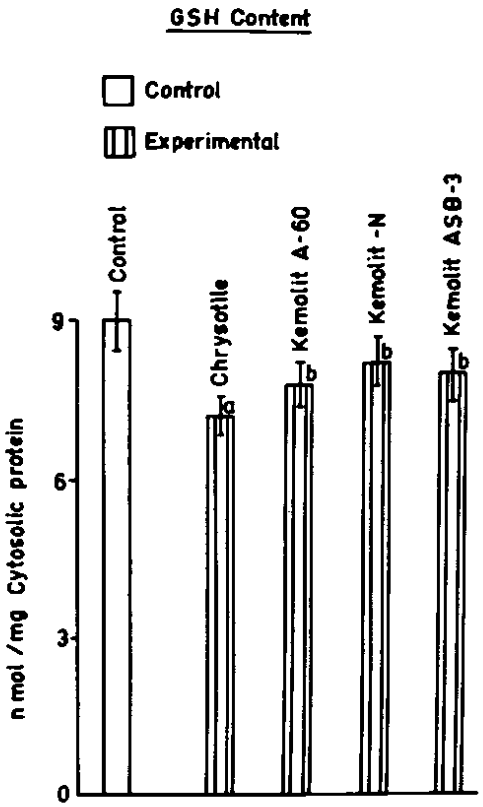


Figure 7. Benzo(a)pyrene hydroxylase activity in lung microsomes, isolated from control and dust treated rats. The values represent mean ± SEM of six animals $^{a}p < 0.001$, $^{b}p < 0.02$, $^{c}p$ – N.S.

Table III

Lipid Peroxidation in Control and Dust Treated Animals

| Treatments | Control | Chrysotile | Kemolit A-60 | Kemolit N | Kemolit ASB-3 |
|---|---|---|---|---|---|
| Microsomes | 0.28±0.022 | 0.374±-.018$^{a}$ | 0.361±0.024$^{a}$ | 0.346±0.032$^{b}$ | 0.295±0.038$^{d}$ |
| Microsomes + NADPH | 1.28±0.12 | 2.154±0.16$^{a}$ | 2.054±0.13$^{a}$ | 1.73±0.18$^{c}$ | 1.55±0.14$^{d}$ |
| Microsomes + $Fe^{++}$ | 4.68±0.23 | 7.041±0.52$^{a}$ | 6.753±0.45$^{a}$ | 5.85±0.38$^{b}$ | 5.12=0.43$^{d}$ |

The values are expressed as mean ± SEM of six animals.

$^{a}p < 0.001$; $^{b}p < 0.02$; $^{c}p < 0.05$; $^{d}p$-N.S.

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Figures 8-11 not provided

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# II


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