INTERACTION BETWEEN FATTY ACID SALTS AND THE ELASTIN NETWORK

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1

General Introduction

Elastin, a network of polypeptide chains, is responsible for the elastic properties of blood vessel walls and connective tissues. It has a strong tendency to associate with various amphiphilic substances, such as fatty acids, bile acids, cholesterol and other lipoitic materials[1-9]. Binding of these substances to the elastin of the arterial intima probably is one of the earliest stages in the formation of atherosclerotic plaque. Claire et al.[5] found a large accumulation of free, i.e. nonesterified, fatty acids in atherosclerotic lesions. Kagan and coworkers[1,3,4] performed in vitro experiments, showing that elastin with fatty acids and bile acids are more susceptible to enzymatic proteolysis than elastin itself. This implies that these substances enhance the degradation of elastin. Both binding of fatty acids and enzymatic degradation of elastin promote a decrease in elasticity of the elastin. As a result, the pulsating ability of the vessel wall decreases. For these reasons, it would be of greatest interest to study the interaction between lipoitic material and elastin. In this study we investigated the (competitive) absorption of sodium salts of fatty acids as a function of their chain length and the influence of this absorption on the elastic properties of elastin.

1.1 Elastin

Elastin consists of a random network of polypeptide chains, which contains mainly (95%) hydrophobic amino acid residues[10]. The

polypeptide chains are interconnected by covalent cross-links. The oxygen and nitrogen atoms in the polypeptide chain form hydrogen bridges, which makes elastin hard and brittle. To acquire rubber elasticity, elastin requires water or another polar solvent to break down the hydrogen bridges.

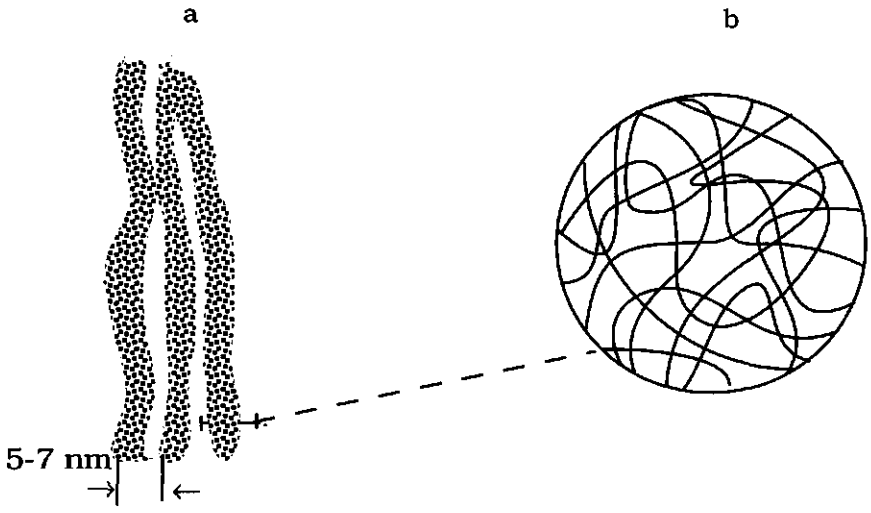

Figure 1.1 Schematic illustration of the architecture of an elastin strip (a) Elastin is made up of a bundle of filaments. (b) Cross-section through one filament, showing a network of polypeptide chains

Light and electron microscopic investigations have shed light on the morphology of elastin. Elastin from bovine ligamentum nuchae consists of 5 to 8 μm diameter fibers that run essentially parallel to the long axis[11]. In figure 1.1 a schematic illustration of the architecture of an elastin fiber is given. According to Gotte et al [12], an elastin fiber contains filaments of 3 to 4 nm diameter, having an average separation between their centers of two adjacent filaments of 5 nm. The filaments are arranged approximately parallel to the fiber axis. Similar conclusions were drawn by Pasquali-Ronchetti et al [13] and Forniers et al[14] Ruggeri et al[15] concluded from freeze etching that elastin consist of filaments of 6 to 7 nm, which become roughly parallel to the axis of the fiber upon stretching.

The polypeptide chains in elastin contain 3 to 8 cross-links per 1000 amino acid residues [16]. The chains between two cross-links comprise approximately 70-80 amino acid residues[17,18]. In figure 1.2 the structure of the most important cross-links, desmosine and isodesmosine are shown [19]. The cross-links are formed by reaction of 4 lysine residues [20-23], catalyzed by the enzyme lysyl oxidase. The quaternary ammonium group is a very strong base, which can be titrated beyond pH=11.5.

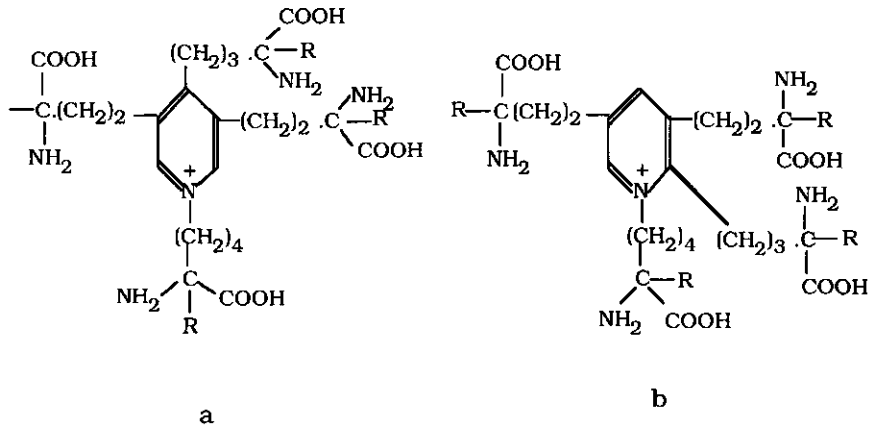

Figure 1.2 (a) Desmosine and (b) isodesmosine. (R is the polypeptide chain)

By analysis of tryptic peptides derived from tropo elastin (precursor of elastin), it was demonstrated that elastin is characterised by the presence of several repeating sequences[23-29]. These repeating sequences make up ±20% of the elastin. The longest and most frequent sequence is the one containing the polypentapeptides valine-proline-glycine-valine-glycine $(VPGVG)_{11}$[30]. This sequence comprises 10% of the total amount of amino acid residues in elastin. Urry et al showed that synthesised $(VPGVG)_{120}$ forms β turns, which, by raising the temperature, coils into helical arrays, called the β spiral [31]. Although β turns possibly occur in elastin, there is no reason to assume that elastin is a non random polymer. On the contrary, the glass transition occurring in elastin[17,18,32-35] can only be explained by assuming random conformational distribution along the polypeptide backbones.

The amino acid analysis of the samples are given in table 1.1

Table 1.1 Amino acid analysis of elastin in residues per 1000 residues

Cysteic acid	5.8
Hydroxyproline	-
Aspartic acid	6.4
Threonine	9.2
Serine	9.1
Glutamic acid	16.9
Proline	121.3
Glycine	330.6
Alanine	223.4
Valine	132.3
Isoleucine	25.2
Leucine	65.2
Tyrosine	7.2
Phenylalanine	31.8
Lysine	4.2
Hydroxy lysine	-
Histidine	0.5
Arginine	6.3
Isodesmosine	1.2
Desmosine	1.6

The majority of the amino acid residues are non-polar. The fraction of the number of polar amino acid residues is less than 10%. Because of the relatively hydrophobic nature of elastin, water is a rather poor diluent. The saturation with water amounts to 0.5 g/g dry elastin. The absence of hydroxylysine indicates that the elastin sample is not contaminated with collagen. Proton titrations of elastin at various concentrations of indifferent electrolyte (i.e. KCl) reveal that the point of zero charge is at pH=3.3, i.e. under physiological conditions it is negatively charged.

1.2 FAS

In this study we used sodium salts of naturally occurring fatty acids (FAS) with various chain lengths. Their gross formula and some other relevant properties are summarised in table 1.2. The FAS belong to the association colloids. This implies that beyond a given concentration, the

so-called critical micelle concentration (CMC), the monomers associate to form micelles. At concentrations beyond the CMC, the concentration of free, non associated molecules is essential equal to the CMC. Values of the CMC are included in table 1.2.

Table 1.2 Relevant properties of FAS used in this study

sodium salt	gross formula	Mol. weight (g/mol)	CMC* (mol/dm^3)
lauric acid	$C_{11}H_{23}COO^-\ Na^+$	222	$2 \cdot 10^{-2}$
myristic acid	$C_{13}H_{27}COO^-\ Na^+$	250	$2.9 \cdot 10^{-3}$
palmitic acid	$C_{15}H_{31}COO^-\ Na^+$	278	$3.9\text{-}6.5 \cdot 10^{-4}$
oleic acid	$C_{17}H_{31}COO^-\ Na^+$	304	$1.4 \cdot 10^{-3}$

*The CMC values refer to 70°C and high pH. They are obtained from tables published by Mukerjee and Mysels [36] (In some cases the values were derived by extrapolating from data given at other temperatures).

The diameter of a FAS micelle can be estimated by calculating the volume of a micelle, using the number of monomers in a micelle, i.e. ca. 80 and the volume per CH_2 group, which is $30 \cdot 10^{-30} m^3$. It follows that the diameter of a micelle varies between 4 and 6 nm, depending on the chain length of the FAS. The pores in the elastin filament are smaller (see figure 2.1). This implies that the micelles can not enter the elastin filament.

To achieve good solubility of the FAS, the experiments were performed under alkaline conditions, i.e. in borate buffer of pH=10, in which the ionic strength is adjusted to 0.15M, unless otherwise stated.

1.3 Outline of the present study

In chapter 2 several experiments are discussed, which give insight into the interaction mechanism between elastin and FAS. Attention will be paid to the absorption kinetics, whereby the short (up to 10h) and long term (10h to several days) absorption will be distinguished. In the short term experiments we attempt to characterise the interaction

mechanism between elastin and FAS. In the long term absorption, the uptake of FAS is coupled with degradation of elastin. The influence of the chain length of the FAS and of the pH of the solution on this degradation is determined. The affinity of the various FAS for elastin is investigated from binding isotherms, whereby degradation has to be taken into account. FAS absorption is accompanied by absorption of solvent, which leads to swelling of elastin. The uptake of FAS, together with the swelling, is followed as a function of NaCl concentration; This enables calculation of the osmotic pressure between the elastin network and the solution.

Elastin behaves as a rubber, which implies that the chains between the cross-links in the elastin network are in rapid motion. This is only possible if a large number of conformations are available to the peptide units in the polypeptide chains. When an externally applied stress is imposed, the chains between the cross-links are stretched, thereby reducing the number of possible conformations. This results in a retractive force. Hence, the elasticity of elastin has an entropic nature. In chapters 3, 4 and 5, the influences of the FAS on the elastic properties of elastin are discussed.

In chapter 3 we consider the influence of the FAS on the elasticity modulus, which is the stress divided by the strain. Attention is paid to the direct (caused by the mere presence of FAS in the network) and the indirect (changes of the network caused by the absorption of FAS) influences on the elasticity. Both aspects are investigated with static and dynamic modulus measurements.

In chapter 4 we discuss changes in conformational distribution of the polypeptide chains between two cross-links in elastin due to FAS absorption. The allowed conformations of the chains depend on the rotational freedom of the chain bonds, which, in turn, depend on the size of neighbouring groups and the rotational freedom of neighbouring bonds. In view of this, it might be expected that the conformational distribution of the chains changes upon FAS absorption. These changes are detected by static elasticity and thermo-elastic measurements.

In chapter 5, the influence of the FAS on the rubber-glass transition is discussed. Upon lowering the temperature, the number of accessible rotational states around a chain bond decreases as a result of decreasing thermal energy. Ultimately, over a small temperature

interval, rotational transitions become rare within the time of measurement. The sample looses its thermo-elastic behaviour and a stiff, brittle structure results, which is the glassy state. The glass transition is characterised by a change in the heat capacity of the sample. As the number of accessible conformations of a chain depends on its environment, binding of FAS may influence the transition from rubber into glass. This transition of elastin and elastin-FAS complexes were measured by following the heat capacity of the sample.
In the summary, an overview of the results, concerning the absorption of FAS in elastin, and its influence on the elasticity is given.

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2

Absorption of FAS in elastin

2.1 Introduction

In this chapter we describe the absorption of fatty acids salts (FAS) in elastin with view to characterise the interaction mechanism. Elastin is an hydrophobic polypeptide, which implies that the interaction between FAS and elastin is at least partly of hydrophobic nature[1,2]. Therefore we investigated the influence of the apolar chain of the FAS on absorption by varying the chain length.
Attention will be paid to the absorption kinetics, whereby short (up to 10h) and long term (10h to several days) absorption will be distinguished because different physical processes occur. For instance, upon long exposure of elastin to FAS solution, degradation of elastin occur. The influence of the chain length of the FAS and the pH of the solution on this degradation is discussed. Because the absorption of FAS is reversible, the affinity of the FAS for elastin can be determined by calculating the standard Gibbs energy from the binding isotherms.
The absorption of FAS is accompanied by absorption of solution. The uptake of FAS together with the swelling is followed as a function of NaCl concentration in solution. This enables calculation of the osmotic pressure between the elastin network and the solution, giving insight in the degradation mechanism of elastin during absorption.

After studying the absorption mechanism, the influence of FAS on the rubber-elastic behaviour will be discussed in the following chapters.

2.2 Methods

2.2.1 Absorption as a function of time

Elastin was purified from bovine ligamentum nuchea obtained at a local slaughterhouse. The procedure was described by Patridge et al.[3]. The samples were scraped free from extraneous tissue, cut into thin strips and extensively rinsed with distilled water. The strips were autoclaved twice for 6h at 120°C and thoroughly washed again. Then they were dried at 80°C to constant weight and stored over silica in a dessicator.
The absorption experiments of the fatty acid salts (FAS) in elastin have been performed under alkaline conditions at a temperature of 70°C in order to achieve good solubility of FAS. For the same reason all solutions have been made in borate buffer of pH = 10 in which the ionic strength was adjusted to 0.15M by adding NaCl.

Table 2.1 Survey of the absorption experiments.

absorption type	FAS	concentration	elastin per sample	solution per sample
short absorption time				
single	laurate myristate palmitate	2, 5, 10 and 20 g/dm^3	250 mg	100 cm^3
competitive	lau-myr lau-pal myr-pal	5 g/dm^3 of each FAS	250 mg	100 cm^3
long absorption time				
single	laurate myristate palmitate	5,10 g/dm^3	100 mg	50 cm^3
competitive	lau-myr lau-pal myr-pal	5 g/dm^3 of each FAS	100 mg	50 cm^3

The experiments are divided in short (up to 10 hours) and long term absorption experiments (up to 200 hours). The different types of FAS

and the mixtures that were studied, are given in table 2.1. Both single component absorption as well as competitive absorption have been studied.
The experiments were carried out in the following way: Samples of dry elastin, having a length of approximately 5 mm and a cross section of approximately 10 mm^2, were immersed in buffer solutions at 70°C. After two hours, when the elastin was saturated with buffer, the elastin samples were transferred to the buffered FAS solutions. The amounts of elastin and the volumes of the solutions are, for each experiment, given in table 2.1.
After various incubation times, samples were removed from the solutions, dried and weighed in order to determine the amount of absorbed FAS as a function of time. The experiments were performed in duplicate.

In the competitive absorption experiments, the ratios of the different absorbed FAS were established together with the total FAS absorption. This has been done by gas chromatography. To this end, the FAS were methylated according to the following procedure: First the FAS were desorbed from elastin by transferring the elastin-FAS complex to water at 70°C. To check if all FAS were desorbed, the mass of elastin before absorption was compared with that after desorption. The methylation required a water-free environment. This was achieved as follows. First a few drops of concentrated H_2SO_4 (98%) and one part of methanol was added to one part of FAS in water. H_2SO_4 converted the FAS into fatty acids. One part of this solution was extracted with two parts of hexane. After extraction, all FAS were in the hexane phase in the form of fatty acids. The hexane phase was separated from the water phase and dry fatty acid residues were obtained by evaporation of the hexane in a rotation vaporiser.
For the methylation, 4 cm^3 0.5N methanolic KOH was added to the residue. The mixture was boiled for 5 to 10 minutes under reflux, to dissolve the residue. Then, 5 cm^3 14% BH_3 in methanol was added through the refluxer, followed by boiling during 2 minutes. Subsequently 2 to 3 cm^3 hexane were added and the solution was boiled for another minute. The receiver flask was taken from the heater and a saturated aqueous solution of NaCl was added till the hexane

phase was in the neck of the receiver. After this, an exactly weighed amount of margaric acid methyl ester dissolved in 2 cm^3 hexane, used as an internal standard, was added to the hexane phase. Finally, the hexane phase was separated from the saturated NaCl solution and dried over Na_2SO_4. The amount of each fatty acid in the sample was determined by gas chromatography[4].

2.2.2 Absorption as a function of FAS concentration

The method to determine the absorption as a function of FAS concentration is similar to the method described in the preceding section. Absorption also took place under alkaline conditions (pH 9, 10 and 11) and at 70°C. Strips of elastin were cut into small pieces and about 100 mg of dry elastin was immersed in 50 cm^3 FAS solutions of varying concentrations. In order to test the influence of the nature of the polar head group of the surfactant on its affinity to bind to elastin, hexadecane sulfonate has also been included in the experiments. After incubation times of 10 h, 3 or 7 days, elastin was removed from the solution and the absorbed amount determined gravimetrically.
Elastin with absorbed FAS was transferred to pure water at 70°C for one or two days, to desorb FAS from the elastin. To check reversibility, the mass of elastin before absorption was compared with that after desorption

2.2.3 Absorption as a function of c_{salt}.

Strips of approximately 8 cm length and cross section of 10 mm^2 were immersed in buffer solution. After 4 hours, when the strips were saturated with buffer, they were removed from the solution and the lengths were determined. Next, the strips were immersed in 10 g/dm^3 FAS solution of various ionic strengths. As in most experiments, the FAS solutions were prepared in buffer of pH = 10. The amount of NaCl which was added to the buffer varied between 0 and 0.20 mol/dm^3. After various incubation times, the strips were removed from the solutions, after which the length was measured.
The amount of absorbed FAS was also determined, by weighing dried strips before and after FAS desorption respectively. Prior to re-

immersing the strips in the FAS solutions, they were moistened in steam, to re-adjust the volume to the value before drying. Then the procedure was repeated.

2.2.4 Degradation of elastin caused by FAS absorption

Strips of elastin of about 8 cm length and cross section of 10 mm^2, were immersed in buffer solutions. After reaching equilibrium the lengths of elastin strips were determined. Then, the strips were transferred to FAS solutions of 10 g/dm^3. After an incubation time of approximately 2 days, the strips were re-immersed in pure water of 70°C in order to desorb the FAS. After desorption the length of the strip was measured. The strip was re-immersed in the FAS solution and the whole procedure was repeated at various incubation times.

2.3 Results and discussion

2.3.1 Absorption as a function of time

2.3.1.a Single component absorption: Short incubation time

In figure 2.1 the absorption of FAS from solutions of various concentrations is shown as a function of time during the first 10 hours of incubation. During the first two hours, the amount of FAS in elastin increases very fast. Thereafter the absorption rate decreases. It appears that, for each FAS, the absorption increases with concentration, even for concentrations above the critical micelle concentration (see table 2.2). it continues to increase.

Table 2.2 CMC's of the FAS [5]

FAS	CMC
laurate	4.4 g/dm^3
myristate	0.7 g/dm^3
palmitate	0.2 g/dm^3

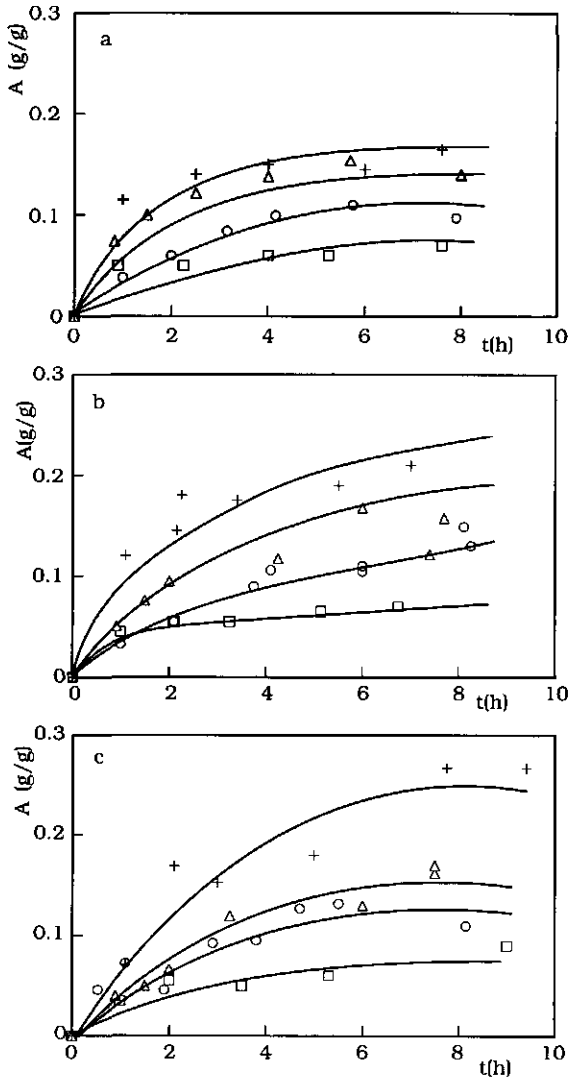

Figure 2.1
Uptake of a) laurate, b) myristate, c) palmitate in solutions with
(□) 2 g/dm^3
(○) 5 g/dm^3
(Δ) 10 g/dm^3
(+) 20 g/dm^3

In chapter 1, it has been argued that micelles of FAS cannot enter the network. This implies that only free FAS molecules can bind into elastin. Although it remains possible that bound FAS molecules associate with each other. It might be expected that the absorption does not substantially increase with increasing concentration beyond the CMC, because in that case the concentration of free FAS is

approximately constant. However, FAS absorption in elastin continues to increase beyond the CMC. So the driving force is the difference in the affinity of the monomers for micelles and elastin. It is concluded that, above the CMC, FAS molecules bind to elastin rather than to each other in a micelle. Hence, the micelles may be considered as reservoirs from which monomers are released to become bound to elastin.

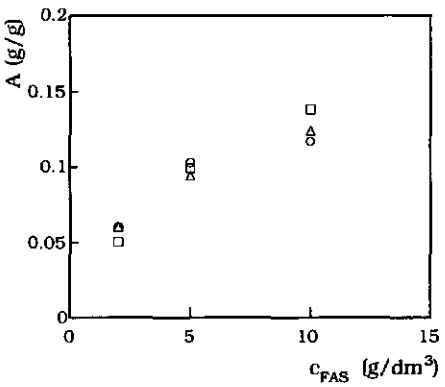

Figure 2.2 The uptake of (□) laurate, (○) myristate, (Δ) palmitate after an incubation time of 4 h

In figure 2.2 the amounts absorbed after four hours of incubation are given as a function of FAS concentration in solution. The figure shows that the variation in the absorbed amounts of the different FAS molecules is very small, especially at concentrations below 4.4 g/dm^3. This is an interesting result. In the low concentration range laurate is present in solution as monomers, whereas myristate and palmitate molecules are mainly present in micelles. It supports the conclusion of the preceding paragraph that micelles disaggregate and that monomer FAS molecules are transported into the elastin network.

Absorption kinetics

The simplest mechanism, which could possibly account for the observed, is based on the assumption that there are absorption sites, that these sites are equivalent and that the ability of the FAS molecule to bind is independent of whether or not neighbouring sites are occupied. Such a mechanism may be expected to apply in the initial stages of uptake. In later stages FAS may also attach to other molecules that are already bound, to form aggregates. The validity of such an initial mechanism can be explored by investigating the influence of the

concentrations of reactants on the absorption rate. It seems plausible that the rate is dependent on the concentration of FAS in the solution within the elastin network and the number of available absorption sites. The absorption rate can be expressed as the following second order differential equation:

$$\frac{dA}{dt} = k_a c_{FAS} (X - A) - k_d A \qquad [2.1]$$

where X is the number of absorption sites in mol per gram elastin and A the number of sites occupied by FAS molecules (also expressed in mol/g). k_a and k_d are constants for the rates of absorption and desorption, respectively. By comparing the diffusion coefficient of various substances[6,7], the diffusion coefficients of a FAS monomer in the network is estimated to be of the order 10^{-9}-10^{-10} m^2s^{-1}. It takes 0.3 to 3 h to saturate an elastin strip with cross-sectional area of 10 mm^2. Equation 2.1 counts for that part of elastin, that contacts the saturated FAS-solution. The concentration of FAS, c_{FAS}, in the network is considered to be the same as the concentration in bulk solution. During each uptake experiment the bulk concentration remains approximately constant.

Equation 2.1 may be rewritten as

$$\frac{dA}{dt} = k_a c_{FAS} X - A\left(k_a c_{FAS} + k_d\right) \qquad [2.2]$$

Here, $k_a c_{FAS} X$ and $k_a c_{FAS}+k_d$ are constants, for which we write k_1 and k_2, respectively:

$$\frac{dA}{dt'} = k_1 - k_2 A \qquad [2.3]$$

Defining $y = k_1 - k_2 A$, so that $A = \frac{k_1 - y}{k_2}$ and $dA = -\frac{1}{k_2} dy$, we obtain:

$$\int_{y(A=0)}^{y(A)} \frac{dy}{y} = \int_0^t -k_2 \, dt' \qquad [2.4]$$

which gives upon integration:

$$\ln\frac{y(A)}{y(A=0)} = -k_2 t \qquad [2.5]$$

or, in terms of k_a and k_d:

$$\ln\frac{k_a c_{FAS} X - A\left(k_a c_{FAS} + k_d\right)}{k_a c_{FAS} X} = -(k_a c_{FAS} + k_d)\, t \qquad [2.6]$$

Substituting k_a/K for k_d, we obtain:

$$\ln\left(1 - \frac{A}{X}\left(1 + \frac{1}{K\, c_{FAS}}\right)\right) = -\left(k_a\, c_{FAS} + \frac{k_a}{K}\right)t \qquad [2.7]$$

This second order absorption mechanism is tested by applying equation 2.7. To that end, values for X, K and k_a are needed.
An estimation of X will be given in section 2.3.2, table 2.3 of this chapter. It amounts to 0.6 ± 0.2 mmol per gram elastin. In that section it will also be shown that during long term absorption X increases. However, during the first 10 hours of incubation X does not change significantly, and this is the condition presently under consideration.
The relatively high affinity between FAS and elastin implies that $k_a >> k_d$. From affinity measurements, to be discussed in section 2.3.2 of this chapter, values for k_a/k_d are determined. They are 110 ± 30, 250 ± 50 and 405 ± 80 dm^3/mol for laurate, myristate and palmitate, respectively. It means that at a concentration of 10 g/dm^3, $k_a c_{FAS}/k_d$ amounts to 5 ± 1 for laurate, 10 ± 2 for myristate and 15 ± 3 for palmitate. At the beginning of the absorption process X-A is much larger than A. Hence, especially in the case of high concentrations and in the initial stages, the desorption term $k_d A$ on the right hand side of equation 2.2 may be neglected, so that under these conditions, equation 2.2 simplifies into:

$$-\left[\frac{\mathrm{d}(X-A)}{\mathrm{d}t}\right]_{t\to 0} = \left[\frac{\mathrm{d}A}{\mathrm{d}t}\right]_{t\to 0} = k_a\, c_{FAS}\,(X-A) \qquad [2.8]$$

which allows evaluation of k_a.
Integration of equation 2.8 gives:

$$\ln\frac{(X-A)}{X} = -k_a\, c_{FAS}\, t \qquad [2.9]$$

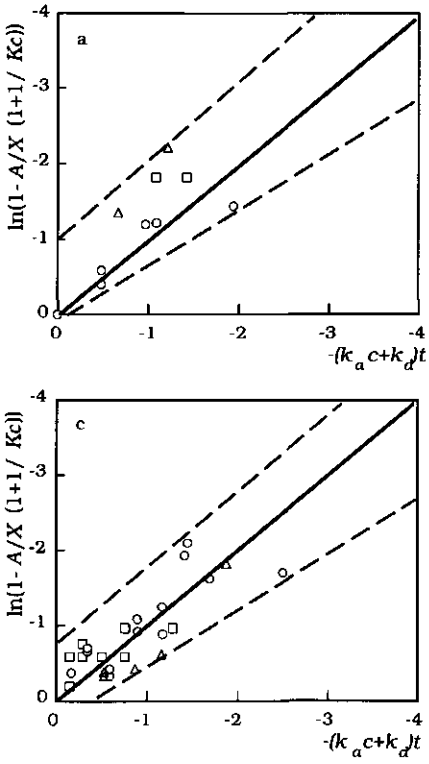

Figure 2.3
Plots according to equation 2.8. On the ordinate and abscissae axis the l.h.s. and r.h.s. of [2.8] are plotted a) laurate, b) myristate, c) palmitate. Dashed: borders of the reliability interval. Different symbols refer to different concentrations.

Estimates for k_a can be obtained from the initial slopes of the plots $\ln(X\text{-}A)/X$ versus time. The values thus obtained for k_a appear to be ca. 8 dm^3/mol h for all three types of FAS. This value is a first and crude approximation, which can be improved by finding the best fit of equation 2.7; this has been done by iteration using 8 dm^3/mol h as the starting value for k_a. The best values for k_a appeared to be 15 ± 5 $dm^3\ mol^{-1}\ h^{-1}$ for laurate, 20 ± 4 dm^3/mol h for myristate and 15 ± 4 dm^3/mol h for palmitate. Hence, the k_a-values for the different FAS

overlap each other, so that no significant difference between the k_a-values for the respective FAS can be established.
Figure 2.3 shows plots where on the ordinate and abscissae axes, the left and right hand side of equation 2.8 are plotted, respectively. According to this equation, the data points should obey a straight line bisecting the origin; this appears more or less the case for all types of FAS. The approximated values for k_a and K are subject to relatively large uncertainties. Based on the experimental data these are ca. 20% both for k_a and K (see table 2.4 in section 2.3.2). The corresponding reliability interval is marked by the dashed lines in figure 2.3. All data points fall within this, relatively wide, interval. Together with the finding in section 2.3.2 that the absorption equilibrium can be described by the Langmuir equation (which follows from equation 2.2 in the case that $dA/dt = 0$), we conclude that the hypothesis of second order kinetics for the binding of FAS molecules to elastin is acceptable.
This type of kinetics is at the basis of the interpretation of experimental results of competitive absorption from mixtures of different FAS.

2.3.1.b Competitive absorption: Short incubation time

The results presented in figure 2.2 suggest no significant differences between the absorption affinity of the three FAS. However, absorption competition between the different FAS is a more sensitive tool to investigate preferential absorption. In this way, the effect of hydrocarbon chain length on the absorption affinity is investigated.

Absorption from mixtures as compared to absorption from single component solutions.
As in the single FAS absorption studies, the total concentration of FAS in solution is 10 g/dm^3, i.e. 5 g/dm^3 for each FAS. The total amount of absorbed FAS from mixtures does not differ much from that for single FAS absorption from a solution with the same total concentration (see figure 2.4). This is expected because the variation in single FAS absorption is small between laurate, myristate and palmitate.
If second order binding kinetics applies, as described in the previous section, it is expected that in a competition experiment each type of FAS absorbs with a lower rate as compared to absorption from single

component solution of the same concentration of that FAS, i.e. 5 g/dm^3, because the absorption depends on the number of empty sites. In competition the number of empty sites decreases not only by absorption of the one FAS but also by that of the other.

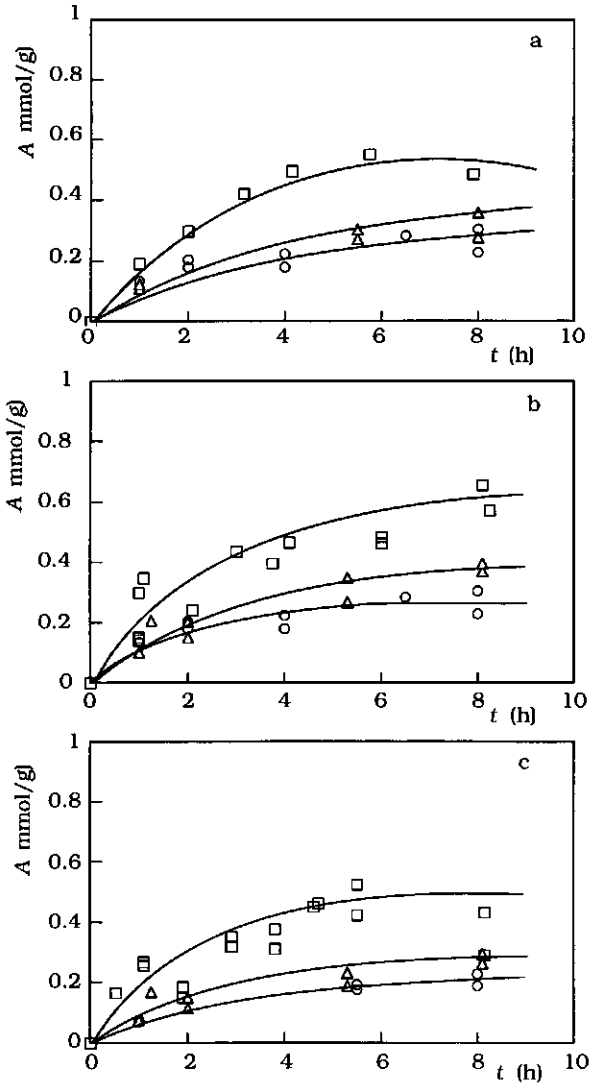

Figure 2.4
Comparison of the uptake of a) laurate, b) myristate and c) palmitate in elastin in (□) single component solutions (10g/dm^3) and in (○), (Δ) mixtures (each 5 g/dm^3)
a) (○)-la-my , (Δ) la-pa
b) (○)-la-my , (Δ) my-pa
c) (○)-la-pa, (Δ) my-pa

In figure 2.4 the amount absorbed of one FAS in competition is compared with that for the same FAS in single component absorption of

concentration 10 g/dm^3. The figure shows that the amounts of FAS absorbed in competition are indeed less than those obtained from single component solutions.

Preferential absorption

In figure 2.5 ratios of FAS absorbed from mixtures are given as a function of time.

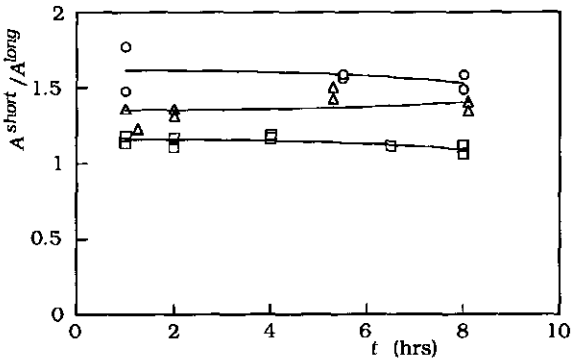

Figure 2.5
Ratio of absorbed FAS in mixtures as a function of time
(□) laurate/myristate
(Δ) myristate/palmitate
(○) laurate /palmitate

In none of the cases does the ratio A^{short}/A^{long} significantly depart from a constant value, from which it is concluded that at least during an incubation time of 8 hours one type of FAS does not displace the other. It is then plausible to assume that each type of FAS absorbs according to a second order mechanism.

During the first hours of incubation the absorption is so small that desorption can be neglected. Then, the absorption rate can be estimated by applying equation 2.8. The ratio of the absorption rates from a FAS mixture is:

$$\frac{dA^{short}/dt}{dA^{long}/dt} = \frac{k_a^{short} c^{short}[X - A^{short} - A^{long}]}{k_a^{long} c^{long}[X - A^{short} - A^{long}]} \approx \frac{k_a^{short} c^{short}}{k_a^{long} c^{long}} \qquad [2.10]$$

The concentrations are $2.3 \cdot 10^{-2}$, $2.0 \cdot 10^{-2}$ and $1.8 \cdot 10^{-2}$ mol/dm^3 for laurate, myristate and palmitate, respectively.

The ratios of absorption rates can be estimated from the initial slopes of the curves given in figure 2.4. These ratios are 1.3, 1.5 and 1.9 for laurate/myristate, myristate/palmitate and laurate/palmitate,

respectively. Thus the smaller the molecules the higher the absorption rates. The corresponding values for k_a^{short}/k_a^{long} are 1.1, 1.4 and 1.5, implying that the shorter FAS molecules have a larger k_a-value than the longer ones. The most probable explanation is that the shorter FAS experience less steric hindrance to bind in the elastin network.
Thus the competitive absorption experiments are indeed more discriminative with respect to preferential absorption of the one FAS over the other than comparison of the results of single FAS absorption.

2.3.1.c Long incubation times

In this section results are shown for incubation times from 10 hours up to several days. The long term experiments are discussed separately from the experiments up to 8 hours incubation time, because different physical processes occur. For instance, upon prolonged exposure of elastin to FAS, aggregation of FAS inside the elastin and degradation of the elastin network may take place. These features will be discussed in the sections 2.3.3 and 2.3.4 of this chapter.

Single component absorption
Figure 2.6 shows that for long periods of time, the absorbed amount increases with increasing chain length.

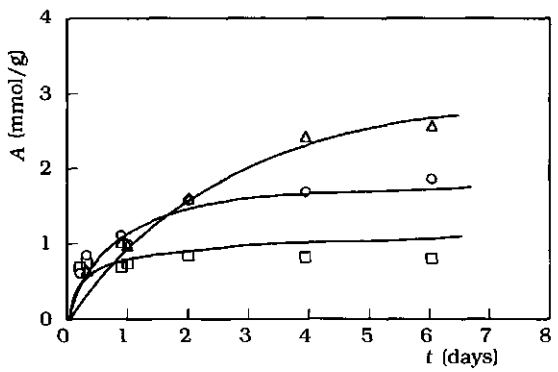

Figure 2.6
Uptake of FAS in solutions of 10 g/dm^3 in the long term experiments
(□) laurate
(○) myristate
(△) palmitate

At short incubation times (≤ 4h) this effect is not yet discernible (compare figure 2.2). It is furthermore shown in figure 2.6, that at least

for myristate and palmitate, absorption tends to increase continually upon prolonged incubation.

Competitive absorption
Absorption from mixtures has also been followed during prolonged incubation periods. In figure 2.7 ratios of absorbed amounts of different FAS are given as a function of time. The values for A^{short}/A^{long} tend to decrease slightly. It points to a very slow displacement of the shorter FAS by the longer ones.

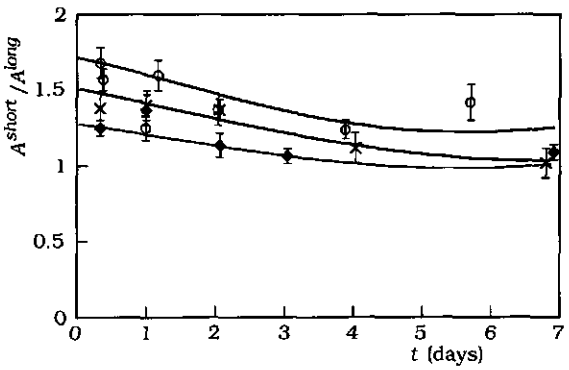

Figure 2.7
Ratios of absorbed FAS in competitive experiments
(♦)laurate/myristate
(×) myristate/palmitate
(O) laurate /palmitate

2.3.2 Absorption as a function of FAS concentration

In the preceding section, absorption as a function of time was considered. The aim of the present section is to discuss the absorption as a function of concentration at long incubation time, i.e.>10 h. All data presented here were obtained in borate buffer of pH = 10 in which the ionic strength was adjusted to 0.15 M by adding NaCl. The pH-dependence will be discussed in section 2.3.4.b

For each FAS, the amount absorbed continues to increase with increasing incubation time. For palmitate, this is illustrated in figure 2.8. The other FAS show a similar trend.
Anticipating the discussion in sections 2.3.3 and 2.3.4, the cross-link density in elastin reduces as a function of time. This reduction leads to an increase in the number of binding sites. Hence, we deal with the sum-effect of two physically distinct processes: a) absorption of FAS

and b) decrease in cross-link density. Process a) reaches equilibrium much faster than process b), so that the absorption of FAS can be treated as a quasi-equilibrium, superimposed on the very slow process of cross-link rupture, of which the non-equilibrium is frozen on the time scale of the absorption process.
Thus, it is assumed that after 10 hours, 3, 7 and 11 days (see figure 2.8) the distribution of FAS between the solution and the elastin network is in equilibrium, but that the number of absorption sites per gram of elastin is different between these incubation times.

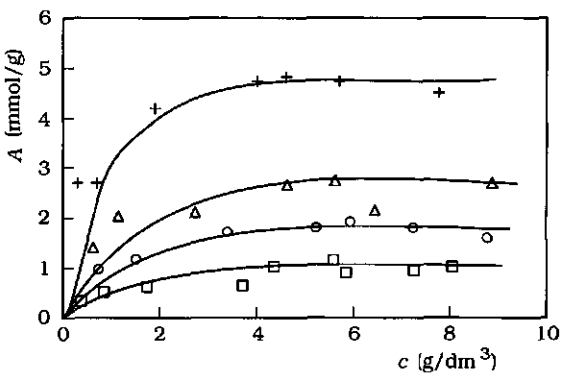

Figure 2.8
Absorption as a function of concentration at 70°C for palmitate at various incubation times
(□) 10 h
(○) 3 days
(△) 7 days
(+) 11 days

At equilibrium the absorption and desorption rates are equal. Hence, equation 2.1 becomes:

$$\frac{dA}{dt} = k_a\, c_{FAS}\,(X - A) - k_d\, A = 0 \qquad [2.11]$$

Hence,

$$\frac{k_a}{k_d}\, c_{FAS} = \frac{A}{X - A} \qquad [2.12]$$

which is the Langmuir equation. This equation can also be written as

$$\frac{1}{A} = \frac{1}{X} + \frac{1}{KX}\,\frac{1}{c_{FAS}} \qquad [2.13]$$

where K is the equilibrium constant k_a/k_d and c_{FAS} is the total concentration of FAS. The *total* concentration was taken because of the following consideration: In section 2.3.1.a it was explained that the micelles act as reservoirs from which monomers are released to become bound to elastin. The exchange of a monomer between micelles and bulk solution occurs on the time scale of microseconds. This exchange is much faster than the transport to, and the absorption of a monomer, in elastin. Hence, kinetically speaking, a solution with micelles and monomers acts as a solution of monomers in which all the FAS are present as monomers.

The standard Gibbs energy of absorption, $\Delta_{abs}G^0$ is related to the equilibrium constant as

$$\Delta_{abs}G^0 = -RT\ln K \qquad [2.14]$$

According to equation 2.13, the number of binding sites, X, at each incubation time has to be known, to determine $\Delta_{abs}G^0$. The increase of this number can be determined by plotting the maximum absorption, which is the absorption for c > 5 g/dm^3 (see figure 2.8), as a function of time. This is done in figure 2.9. The increase is given by the slope and the intercept is the absorption site density at t = 0. In table 2.3 these values are given for various FAS and for hexadecane sulfonate.

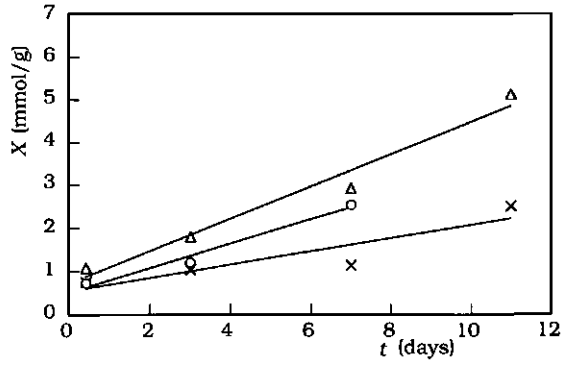

Figure 2.9
Increase in total number of absorption sites in elastin as a function of time in solutions
(×) laurate
(O) myristate
(Δ) palmitate

Table 2.3 shows that the number of absorption sites per gram elastin at $t = 0$ does not differ significantly between the various kinds of FAS. It also demonstrates that the number of binding sites increase more rapidly with increasing hydrocarbon chain length of the FAS. In section 2.3.4, it will be shown that the degradation rate, and hence, the number of absorption sites, increases with increasing chain length of FAS. The deviating behaviour of oleate may be caused by the double bond in the alkyl chain, which imposes steric hindrance when penetrating the elastin network.
Furthermore, the relatively large differences between FAS and hexadecane sulfonate implies that the type of head group plays a role in the absorption process.

Table 2.3 Increase in binding sites

FAS	***X* at $t = 0$·** (mmol/g elastin)	**increase in sites** (mmol/g day)
laurate	0.5 ± 0.3	.05 ± .04
myristate	0.5 ± 0.2	.28 ± .04
palmitate	0.7 ± 0.3	.38 ± .05
oleate	0.5 ± 0.2	.22 ± .02
hexadecane sulfonate	0.2 ± 0.2	.03 ± .01

Equilibrium constants are determined by plotting $1/A$ against $1/c$. In table 2.4 these constants and the corresponding standard Gibbs energies are given. It is clear that the standard Gibbs energy becomes more negative with increasing chain length. This implies that the affinity increases with increasing hydrocarbon chain length, which suggests that the binding is, at least partly, of hydrophobic nature. This dependency of the binding affinity on chain length is in agreement with the displacement of the shorter FAS from the elastin binding sites by the longer ones, as was observed in the long-term incubation experiments (section 2.3.1.c)

The affinity of the head group of FAS can be found by plotting $\Delta_{abs}G^0$ as a function of chain length, see figure 2.10. Extrapolating to zero chain length, yields $\Delta_{abs}G^0$ of the head group, i.e. -3 ± 1 kJ/mol.

Because the affinity of hexadecane sulfonate to bind to elastin is larger than that of the FAS having the same chain length, it is concluded that $\Delta_{abs}G^0$ of the sulfonate group is larger than of the carboxyl group. Apparently not only the charge but also the size of the head group is important. The isoelectric point of elastin is at pH 3.3 (see chapter 1, section 1.1). Hence at pH = 10, at which the experiments are done, elastin is negatively charged. The cross-links of elastin have a quaternary ammonium ion[8], which is positively charged at pH = 10. At this pH, lysine is also positively charged. Hence, the favourable interaction between the negatively charged head-group and elastin may be electrostatic attraction.

Table 2.4 Absorption equilibrium constants and standard Gibbs energies of FAS and hexadecane sulfonate in elastin

FAS	chain length	incubation time(days)	K (dm^3/mol)	average K (dm^3/mol)	ΔG^0 kJ/mol
laurate	C12	0.4	124 ± 30		
		3	114 ± 30	110 ± 30	-13.4 ± 0.8
		7	90 ± 20		
myristate	C14	0.4	264 ± 50		
		3	258 ± 50	250 ± 50	-15.7 ± 0.7
		7	223 ± 50		
palmitate	C16	0.4	380 ± 80		
		3	395 ± 80	405 ± 80	-17.1 ± 0.7
		7	440 ± 80		
oleate	C18	0.4*	612 ± 120		
		3*	720 ± 150	695 ± 150	-18.6 ± 0.7
		7*	757 ± 150		
hexa-decane sulfonate	C16	0.4*	730 ± 150	>700	< -19
		3*	1050 ± 150		

All measurements were carried out in duplicate except those marked with *

The slope of the line in figure 2.10, gives -0.8 ± 0.2kJ/mol for the ΔG^0-increment per CH_2 group. This value is much smaller than the value of -4 kJ/mol, reported for the transfer of one mole of -CH_2- from an aqueous solution to an apolar environment[9]. A plausible explanation is that a FAS monomer from solution that binds to the elastin is replaced by a FAS monomer released from a micelle, so that the net process involves the transfer of FAS monomer from a micelle into the elastin network. The Gibbs energy change of this transport is still negative, meaning that CH_2 groups have a slightly greater affinity for the elastin than for the surfactant micelles in solution.

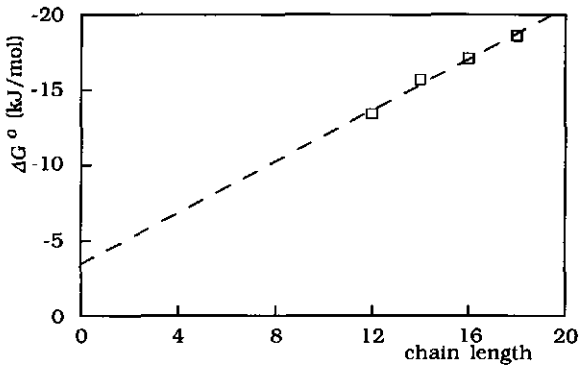

Figure 2.10
ΔG^0 as a function of chain length

2.3.3 Absorption as a function of c_{salt}

In figure 2.11 the uptake of FAS is plotted as a function of c_{salt}. The figure displays, at each incubation time, the same trend: In the case of laurate and myristate, the absorbed amount increases as c_{salt} increases. With palmitate this is also the case for $c_{salt} < 0.1$ M; at higher c_{salt} the dependency shows a rather irregular pattern.

The absorption of FAS is accompanied by swelling of the elastin. Elastin consists of an isotropic network, which implies that from the change in length during absorption, the change in volume can be determined by applying equation 2.15.

$$\left(\frac{L_0 + \Delta L}{L_0}\right)^3 = \frac{V_0 + \Delta V}{V_0} \qquad [2.15]$$

It appeared that this swelling is independent of c_{NaCl}, but increases with absorption time. This is illustrated in figure 2.12 for myristate; laurate and palmitate ($c_{NaCl} < 0.1M$) show the same trend. It seems that the swelling of elastin is restricted by the elasticity of elastin. The increase in swelling with absorption time is caused by the degradation of elastin, that occurs during absorption and which, in turn, decreases the elasticity (see section 2.3.4).

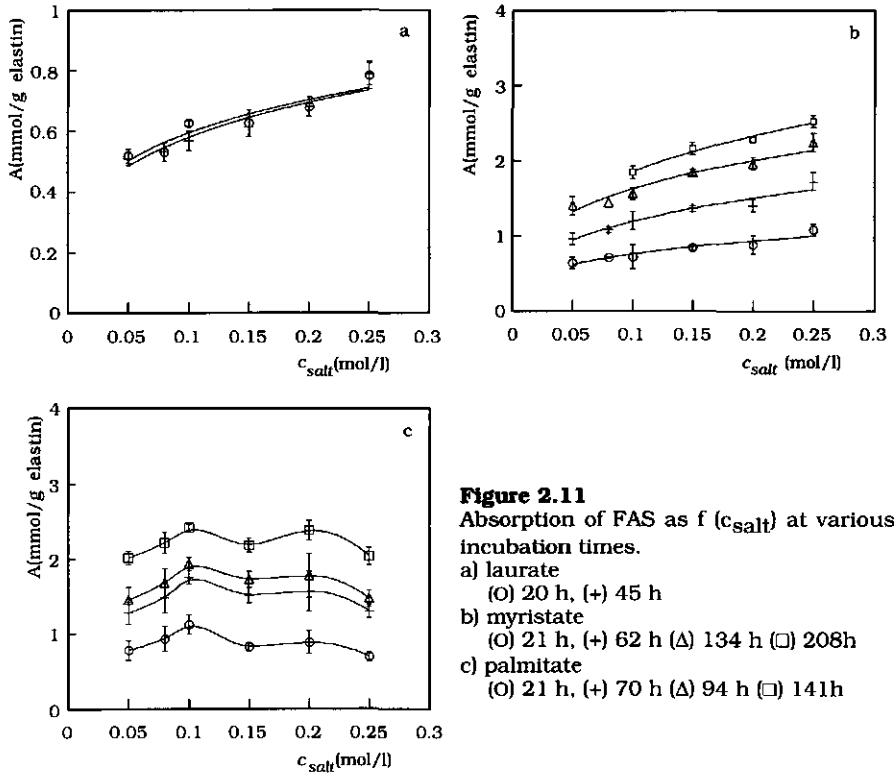

Figure 2.11
Absorption of FAS as f (c_{salt}) at various incubation times.
a) laurate
(O) 20 h, (+) 45 h
b) myristate
(O) 21 h, (+) 62 h (Δ) 134 h (□) 208h
c) palmitate
(O) 21 h, (+) 70 h (Δ) 94 h (□) 141h

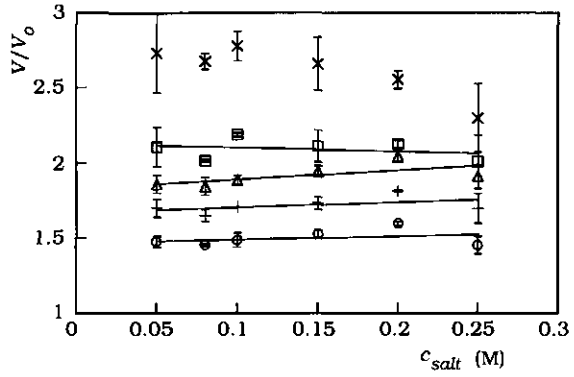

Figure 2.12
Change in volume of elastin in solution of myristate (10 g/dm^3) as a function of c_{salt} at an incubation time of
(O) 21 h
(+) 42h
(Δ) 62h
(□) 82h
(×) 134h

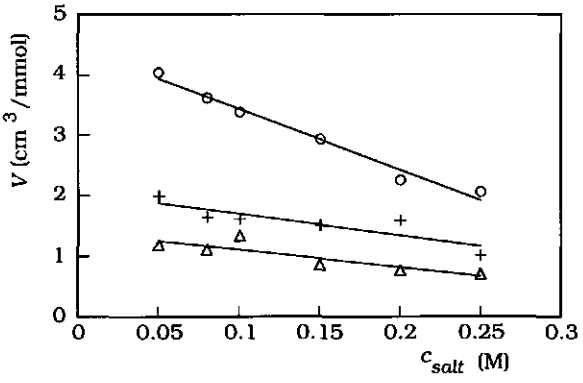

Figure 2.13
Absorbed volume of solution per mmol absorbed FAS
(O) laurate
(+) myristate
(Δ) palmitate

The absorption of FAS and solution (with salt) causes a difference in concentration of ions between the solutions inside and outside the elastin network, resulting in an osmotic pressure between the elastin sample and the surrounding solution. The osmotic pressure can be approximated by using the van 't Hoff equation[10]:

$$\Pi = g R T \Delta c \qquad [2.16]$$

Π is the osmotic pressure. Δc is the difference in concentration of ions inside and outside elastin, whereby the FAS bound to elastin must be taken into account. The few charged groups in elastin may be neglected. g is the osmotic coefficient, which compensates non ideal

behaviour. The difference in concentration in ions between elastin and solution is:

$$\Delta c = 2\left(c_{NaCl}^{el} + c_{FAS}^{el}\right) - 2\left(c_{NaCl}^{sol} + c_{FAS}^{sol}\right) \qquad [2.17]$$

c_{FAS}^{el} was determined by measuring the absorbed amount of FAS in elastin together with the swelling of elastin, due to the uptake of solution, given in figure 2.13. The FAS concentration in solution, c_{FAS}^{sol}, was calculated from the initial concentration and the amount absorbed in elastin. It appeared that c_{FAS}^{sol} did not change significantly during the experiment; c_{NaCl}^{sol} and c_{NaCl}^{el} can be estimated by using the Gibbs adsorption equation [11]:

$$d\gamma = -S^{\sigma}dT + \Gamma_{NaCl}d\mu_{NaCl} + \Gamma_{FAS}d\mu_{FAS} \qquad [2.18]$$

where γ represents the interfacial tension, μ the chemical potential of the component indicated, S^{σ} the surface excess entropy and Γ the amount absorbed per unit area.
Cross-differentiation gives:

$$\left(\frac{\partial A_{FAS}}{\partial \mu_{NaCl}}\right)_{\mu_{FAS},p,T} = \left(\frac{\partial A_{NaCl}}{\partial \mu_{FAS}}\right)_{\mu_{NaCl},p,T} \qquad [2.19]$$

in which the amount absorbed per unit volume, A, is used instead of Γ.
Using $\mu_{NaCl} = \mu_{Na^+} + \mu_{Cl^-}$ and $\mu_{FAS} = \mu_{FAS^-} + \mu_{Na^+}$, we can write in terms of activities a:

$$d\mu_{NaCl} = RTd\ln(a_{NaCl} + a_{FAS}) + RTd\ln a_{NaCl} \qquad [2.20]$$

$$d\mu_{FAS} = RTd\ln(a_{NaCl} + a_{FAS}) + RTd\ln a_{FAS} \qquad [2.21]$$

Substituting [2.20] and [2.21] in [2.19] gives:

$$\frac{a_{NaCl}}{2a_{NaCl} + a_{FAS}}\left(\frac{\partial A_{FAS}}{\partial a_{NaCl}}\right)_{a_{FAS}} = \frac{a_{FAS}}{2a_{FAS} + a_{NaCl}}\left(\frac{\partial A_{NaCl}}{\partial a_{FAS}}\right)_{a_{NaCl}} \qquad [2.22]$$

As shown in figure 2.11, $\partial A_{FAS} / \partial a_{NaCl}$ is positive, which implies that according to [2.22], the NaCl concentration in elastin will increase, as FAS will be absorbed. For unknown reason, palmitate absorption from solutions of c_{NaCl} > 0.1 M deviates from this behaviour. We have not included these measurements in our further interpretation.

Using the activity coefficients of NaCl in water at 70°C[12], and the concentration in solutions, a^{sol}_{NaCl} can be calculated. The activity of FAS is approximated by the concentration, c_{FAS}. Inserting the experimental data, given in figure 2.11 in equation 2.22, $\partial A_{NaCl} / \partial a_{FAS}$, can be determined, from which c^{el}_{NaCl} can be calculated. c^{sol}_{NaCl} was calculated from the initial concentration and the amount of absorbed NaCl.

Substituting the values of the various concentrations in equation 2.17, the osmotic pressure in elastin can be calculated using equation 2.16, whereby the osmotic coefficient of NaCl at 70°C is used[12]. In table 2.5 the calculated osmotic pressures are given for the various FAS. These values are independent of the absorption time. The FAS absorption increases as a function of time. It implies that the uptake of solution and NaCl per absorbed FAS monomer is constant.

Table 2.5 Osmotic pressure Π (N/cm^2) at various NaCl concentrations.

c^{solv}_{NaCl}	laurate	myristate	palmitate
0.05M	80 ± 30	205 ± 70	510 ± 200
0.10M	105 ± 35	225 ± 80	900 ± 400
0.15M	140 ± 45	390 ± 130	
0.20M	200 ± 70	580 ± 200	
0.25M	250 ± 85	590 ± 200	

It appeared that the osmotic pressures can reach very high values, up to 900 N/cm^2 (= 90 atm). For comparison, some bacterial cell walls, can stand 60 to 100 atm[13]. The osmotic pressure is much higher than the retractive pressure of elastin, which varies in the samples under

consideration between 40 and 70 N/cm^2, depending on the water content (see chapter 3). It is likely that the high osmotic pressure is, in part, responsible for the degradation of elastin. Both the osmotic pressure and the degradation rate increase with increasing chain length of the absorbed FAS.
Using c^{el}_{NaCl} the Debye length, κ (= $\sqrt{10\ c\ z^2}$) has been calculated. It appears that the distance over which the electrostatic repulsion ($2/\kappa$) is effective, is in the same range as the distance between two FAS molecules in elastin (ca 0.5-1.5 nm). Hence, probably the absorption of ions is required to compensate the electrostatic repulsion between the negatively charged groups of the absorbed FAS. However, electrostatic repulsion is not the only factor that causes swelling, otherwise the absorbed amount of solution per mmol FAS should be almost the same for each FAS. According to figure 2.13 the swelling decreases with increasing chain length.

2.3.4 Degradation of elastin caused by FAS absorption

Cross-link density
In chapter 3 it will be argued that the cross-link density is proportional with the elasticity modulus. Hence, by determining the change in the elasticity modulus of the elastin strips from which pre-absorbed FAS have been desorbed, the reduction in cross-link density can be inferred. Anticipating this discussion, we now describe the change of the cross-link density upon absorption, as derived from changes in the volume of the elastin samples.
The change in cross-link density is determined by measuring the volume of elastin as a function of time. The degree of swelling of elastin in a solvent is the result of a balance of two effects, (1) the tendency of the solvent and solutes to diffuse into elastin and (2) the tendency of the network to contract. The higher the cross-link density, the higher the elasticity and the smaller the internal swelling is[14]. Hence, the volume of elastin in a given solvent is related to the cross-link density.

A quantitative theory for swelling has been developed by Flory and Huggins[14]. This theory predicts a relation between the degree of swelling in a given solvent and the elasticity modulus of the unswollen

rubber-like material. For small degrees of swelling this relation takes the form:

$$\frac{GV_1}{RT} = \frac{A}{Q^{5/3}} \qquad [2.23]$$

where G is the shear modulus of the unswollen rubber, which is the same as the elasticity modulus measured at small deformations (to be discussed in chapter 3). This modulus is proportional to cross-link density ν/V. Q is the ratio of the volume at equilibrium swelling V to the volume of the dry rubber V_{dry}, V_1 is the volume per gram of the solvent and A is a constant whose value is dependent on the particular rubber-liquid system studied.
As R, T, A and V_1 are independent of G,

$$G = \frac{C}{Q^{5/3}} \qquad [2.24]$$

Combination of the initial modulus of elastin with that after FAS absorption gives:

$$\frac{G_t}{G_{t=0}} = \frac{Q_{t=0}^{5/3}}{Q_t^{5/3}} \qquad [2.25]$$

Substituting $G = k\,\nu\,/V$ (k = constant) and $Q = V/V_{dry}$, we obtain:

$$\frac{\nu_t}{\nu_{t=0}} = \frac{V_{t=0}^{2/3}}{V_t^{2/3}} \qquad [2.26]$$

The volumes of the strips at t = 0 were measured in buffer solution. Subsequently the strips were incubated in solutions of FAS or buffer. After different incubation times t, the FAS were desorbed and the volumes determined again. In figure 2.14 curves for $V_{t=0}^{2/3} / V_t^{2/3}$ are given as a function of the time. The FAS have been desorbed in the elastin. From the slopes, the rate of the change in number of cross-links are calculated. These are given in the second column of table 2.6.

They show that the longer the FAS, the faster is the decrease in the number of cross-links.

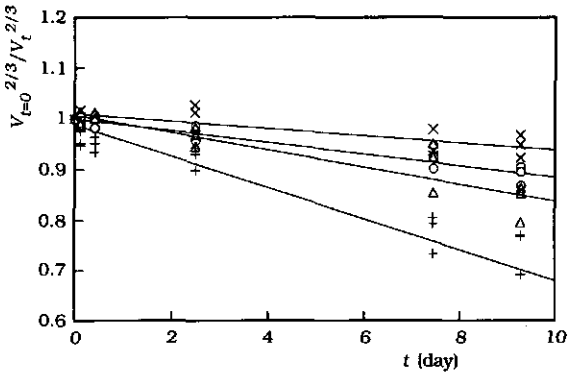

Figure 2.14
Change in volume of elastin from which FAS is absorbed as a function of absorption time
(X) buffer
(O) laurate
(Δ) myristate
(+) palmitate

Table 2.6 Decrease of number of cross-link at T = 343K

FAS	relative decrease in ν per day
none	0.5 ± 0.2%
laurate	1.2 ± 0.3%
myristate	1.8 ± 0.3%
palmitate	3.1±0.3%

In the preceding sections, the absorption as a function of time and concentration were discussed. It appeared that during this absorption, some elastin dissolved in the solution. The dissolved amount is determined by comparing the mass of elastin before absorption with that after desorption of FAS. The rates of elastin dissolution are given in table 2.7, from which it is concluded that the longer the chain length, the larger the rate of elastin degradation.

The correlation between the cross-link reduction and the dissolution of elastin suggests that rupture of cross-links results in degradation of the elastin network, by which fragments of the polypeptide chains are released into the solution. The ratios of the loss of elastin (table 2.7) to the decrease in number of cross-links (table 2.6) are identical for the

various FAS, i.e. 3 ± 2, 2.4 ± 0.5 and 3.0 ± 0.5 for laurate, myristate and palmitate respectively

Table 2.7 Loss of elastin

FAS	loss of elastin (% per day)
laurate	0.4 ± 0.1
myristate	0.75 ± 0.05
palmitate	1.00 ± 0.05

Influence of pH

The influence of pH on FAS absorption was studied for myristate only. In figure 2.15a the absorption's at pH 9, 10 and 11 are plotted as a function of incubation time at a concentrations of 10 g/dm^3.

The figure shows that the absorption depends on the pH; the higher the pH, the higher the absorption rate. In section 2.3.4.a a direct correlation between the number of cross-links and the dissolution of elastin has been indicated. In order to investigate, whether the pH influences the rupture of cross-links, we determined the loss of elastin at various pH values. Figure 2.15b demonstrates that this loss increases with increasing pH. Hence, the concentration of OH^- influences the breakdown of the elastin network, probably through hydrolysis of the cross-links or other bonds in the polypeptide backbone. In general, elastin is known to be strongly resistant to aqueous-alkaline hydrolysis, as is illustrated by a frequently used method for purification[15], i.e. the Lansing procedure, in which elastin is heated to 100°C in 0.1N NaOH. Under normal conditions elastin resists this treatment. However, Robert et al. [16] observed that addition of organic solvents, as aliphatic and aromatic alcohol's, cause a reduced resistance to alkaline hydrolysis. They hypothesised that the hydrophobic interactions between the organic solvent and elastin can open up the densely packed hydrophobic regions of the peptide chains, facilitating the approach of the hydrated OH^- ion to the polypeptide backbone. Absorption of FAS opens up the elastin structure as well. Hence, in case of FAS absorption we may deal with a similar situation as that described for the alcohol's.

The proportionality between the loss of elastin and the amount absorbed is 3.9 $mmol^{-1}$, 3.1 $mmol^{-1}$ and 3.8 $mmol^{-1}$ for pH = 9, 10 and 11. Within experimental error this ratio is constant, i.e. invariant with respect to the pH. This is to be expected, since the increase in FAS absorption as a result of the decreased cross-linking, should not be influenced by the pH.

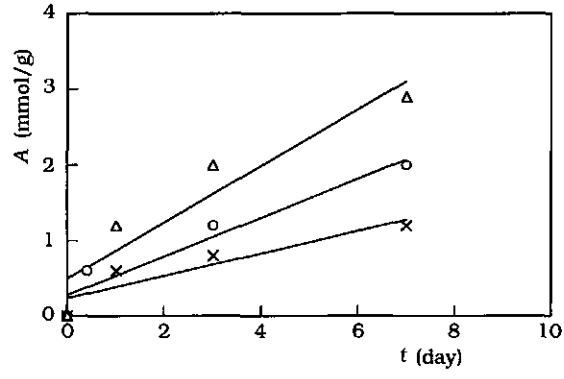

Figure 2.15a
Absorbed amount of myristate as a function of time at
(×) pH = 9
(○) pH = 10
(Δ) pH = 11

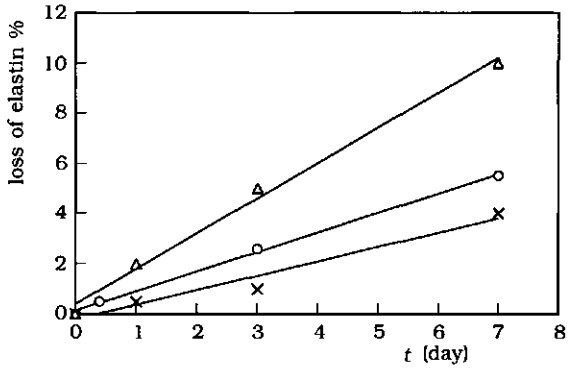

Figure 2.15b
Loss of elastin as a function of absorption time in myristate solution at
(×) pH = 9
(○) pH = 10
(Δ) pH = 11

2.4 Conclusions

The absorption of FAS into the elastin network can be described by a second order binding mechanism, in which the absorption rate depends on the concentration of FAS in solution and the number of absorption sites, and in which the desorption rate depends on the absorbed amount. It appeared that the absorption rate constant decreases with

increasing chain length probably because the shorter FAS molecules are less sterically hindered in the absorption process.
The affinity of the FAS for elastin is mainly of a hydrophobic nature. $\Delta_{abs}G^0$ for the binding of a CH_2 group is -0.8 ± 0.2kJ/mol, which is much smaller then the $\Delta_{abs}G^0$ for the transfer of CH_2 from an aqueous to an apolar environment (-4 kJ/mol), because $\Delta_{abs}G^0$ for the binding of FAS in elastin counts for the transfer of FAS from micelles to elastin. Although elastin is slightly negatively charged at the experimental conditions, the contribution from the negatively charged head group to $\Delta_{abs}G^0$ is exothermic and amounts to about -3kJ/mol. Possibly, the FAS binds in the neighbourhood of the cross-links, which are positively charged at pH = 10.

The binding of FAS is accompanied by absorption of salt and solvent in the elastin. The swelling partly occurs to compensate for the electrostatic repulsion between the negatively charged groups of the FAS in elastin. This swelling and, hence, the absorption of FAS is limited by the cross-linking of elastin. Due to the presence of OH^- ions and absorbed FAS, degradation of elastin occurs, resulting in a decrease in cross-link density and, hence, promoting the absorption of more FAS.
Absorption of FAS causes an osmotic pressure difference between the solutions inside and outside the elastin sample. Both the rate of cross-link rupture and the osmotic pressure is larger for the FAS having the larger chain-length. It suggests that the degradation of elastin is enhanced by the osmotic pressure.

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3

Modulus of elastin

3.1 Introduction

Rubber and rubber-like materials, such as elastin, are characterised by a remarkable combination of two properties. First, they are capable of sustaining large deformations without rupture. Second, the deformed rubber recovers spontaneously to its initial dimensions after removal of the stress. The third important (thermodynamic) feature of rubber is that a deformed piece shrinks when the temperature increases. Correspondingly, stretching of rubber is accompanied by the release of heat[1].

The rubber like properties of elastin can be explained on the basis of its molecular structure. Elastin is made up of a three-dimensional network of cross-linked polypeptide chains. In this network the chains between two cross-links act as statistical chains, which is only possible if large numbers of conformations are available. To achieve this, the chains must be sufficiently long. In a rubber, the various conformational states of the chains all have essentially the same energy. When an externally applied stress is imposed on the network, the chains between the cross-links have to be stretched, resulting in a reduction of the number of available conformations and hence in a decrease of the entropy of the network. Thus, the restoring force has an entropic nature, i.e. it is caused by the tendency of the network to return to its maximum number of conformational states.

As shown in the previous chapter, FAS is strongly absorbed by elastin. It is then likely that the elastic properties changes by absorption of

FAS. The influence of FAS on the elastic modulus is studied with static and dynamic measurements and discussed in this chapter.

3.2 Theory

3.2.1 Thermodynamic analysis

According to the first and second law of thermodynamics for a reversible process:

$$dQ = TdS = dU - dW \qquad [3.1]$$

where dQ is the heat exchange between the system and its surroundings, dS the change in entropy, dU the change in internal energy and dW the work performed on the system. The work can be divided in volume- and stretch-work:

$$dW = pdV + fdL \qquad [3.2]$$

where dL is the length change in one direction at constant volume.
It has already been mentioned that heat is released upon stretching. According to [3.1], this corresponds to an entropy decrease in the rubber network.

Substitution of [3.2] in [3.1], gives the change of internal energy dU, written in terms of variations in entropy S, volume V and length L:

$$dU = TdS - pdV + fdL \qquad [3.3]$$

By combination of [3.3] and introducing the change in Helmholtz energy dF:

$$dF = d(U - TS) \qquad [3.4]$$

we obtain:

$$dF = -SdT - pdV + fdL \qquad [3.5]$$

Thus, for the retractive force f :

$$f = \left(\frac{\partial F}{\partial L}\right)_{T,V} = \left(\frac{\partial U}{\partial L}\right)_{T,V} - T\left(\frac{\partial S}{\partial L}\right)_{T,V} \qquad [3.6]$$

Experimentally it has been proven [2,3,4,5] that by deforming a rubber the change in internal energy $(dU/dL)_{T,V}$ is insignificantly small. Hence, according to equation [3.6], the retractive force is essentially of an entropic nature. It implies that all the work needed for stretching an ideal rubber ($dU/dL_{T,V} = 0$) is used to decrease the entropy of the chains between the cross-links.

3.2.2 Chain statistics

An ideal rubber can be visualised as a loose, three-dimensional network of chains in random conformation, joined together by cross-links. The interactions between the chains are so small, that each chain is assumed to be free to adopt any desired conformation. The resulting motion of such a chain resembles the Brownian motion of a molecule in an ideal gas, superimposed on the constraints, due to the connectivity of the segments. Flory[1,6] used this analogy in order to derive an equation for the change in entropy of a rubber resulting from deformation:

$$\Delta S = \frac{k\,v}{2}\left(\alpha_x^2 + \alpha_y^2 + \alpha_z^2 - 3 - \ln \alpha_x \alpha_y \alpha_z\right) \qquad [3.7]$$

where k is the Boltzmann constant, α is defined as the length L divided by L_i, the length without applied force, whereby the deformations in the x, y and z directions are indicated by the subscripts and v is the number of chains that are involved in the deformation (a chain is the polypeptide chain between two cross-links). In the case of elongation in the x direction, at constant volume, $\alpha_y = \alpha_z = \alpha_x^{-1/2}$, (which implies that $pdV = 0$), equation [3.7] simplifies into:

$$\Delta S = -\frac{k\,v}{2}\left(\alpha^2 + \frac{2}{\alpha} - 3\right) \qquad [3.8]$$

where α equals α_x
As has already been mentioned, for an ideal rubber:

$$f = -T\left(\frac{\delta S}{\delta L}\right)_{T,V} \qquad [3.9]$$

This can also be written as:

$$f = -\frac{T}{L_i}\left(\frac{\delta S}{\delta \alpha}\right)_{T,V} \qquad [3.10]$$

By using

$$\left(\frac{\delta S}{\delta \alpha}\right)_{T,V} = \left(\frac{\delta\left(S_i + \Delta S\right)}{\delta \alpha}\right)_{T,V} = \left(\frac{\delta\,\Delta S}{\delta \alpha}\right)_{T,V}$$

and by combining [3.8] and [3.9], we obtain:

$$f = \frac{vk\,T}{L_i}\left(\alpha - \frac{1}{\alpha^2}\right)_{T,V} \qquad [3.11]$$

Starting point of Flory's theory is that rubber can be treated as a network of individual chain elements, kept together by cross-links, which do not restrict the conformational entropy of the chain elements. However, according to James[7,8] the cross-links cause the motions of the chains to be slightly dependent on each other, a fact that he used in his network theory derived in 1947[7]. Flory[9] gave a short derivation of the network theory of James, which results in a modified equation for the retractive force:

$$f = \frac{vkT}{L_i}\frac{< r_i^2 >}{< r_0^2 >}\left(\alpha - \frac{1}{\alpha^2}\right)_{T,V} \qquad [3.12]$$

$< r_i^2 >$ is the mean square end-to-end distance of the network chains in the isotropic state and $< r_0^2 >$ is the corresponding value for uncross-linked chains in a Θ solvent. The factor $< r_i^2 > / < r_0^2 >$ is a constant (C), which slightly deviates from unity. This factor will be further discussed in chapter 4.

The cross-sectional area (in the y,z plane), A_i, equals V_i/L_i (L_i is the length in the x direction), which, at constant volume, equals V/L_i. Hence,

$$\frac{f}{A_i} = \frac{\nu k T}{V} C\left(\alpha - \frac{1}{\alpha^2}\right) \qquad [3.13]$$

ν / V is the cross-link density. At small deformations of elastin, the force per unit area is, according to Hooke's law[1)], proportional with the deformation:

$$\frac{f}{A_i} = E\frac{\Delta L}{L_i} = E(\alpha - 1) \qquad [3.14]$$

E is a material property, which is called the elasticity modulus. The modulus is a measure of the elasticity. A material is strongly elastic if the modulus has a large value and when E=0, it is purely viscous. Combination of [3.13] and [3.14] gives:

$$E = \frac{\nu k T}{V} C\left(1 + \frac{1}{\alpha} + \frac{1}{\alpha^2}\right) \qquad [3.15]$$

which implies that the elasticity modulus is a linear function of the cross-link density. For small elongation ($\alpha \approx 1$) :

$$E = \frac{3\nu k T}{V} C \qquad [3.16]$$

3.3 Methods

3.3.1 Static modulus measurements

Experimental set up

Using the experimental set-up, illustrated in figure 3.1, the lengths of elastin strips were measured as a function of the applied force. From these measurements elasticity moduli were calculated. An elastin strip of approximately 8 cm length and ca. 0.2 cm^2 cross-section was tightened between two clips and vertically suspended in a cylinder of 250 cm^3 filled with paraffin or aqueous buffer and placed in a water bath, thermostatted at 70°C. Constant forces were applied to the strip by suspending loads from its lower ends. The lengths of the strips were measured with a cathetometer, which was placed at a distance of approximately two meters.

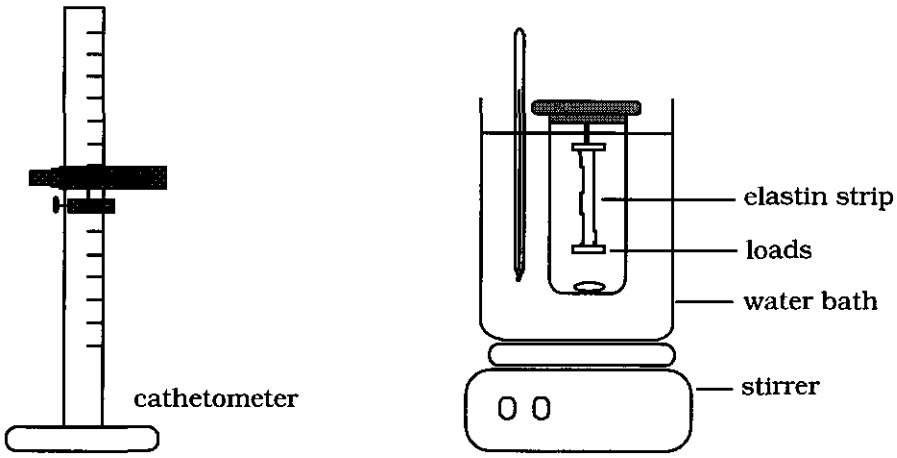

Figure 3.1 Experimental set up, used for the static measurements.

Static modulus determinations were performed to obtain information on the influence of the elasticity of elastin caused by:

a Incubation in buffer of pH = 10 at 70°C

In these experiments, strips were incubated for 0.5, 3 or 7 days in borate buffer at 70°C. After incubation, the strips were removed from

the buffer solution and placed in dessicators of various humidities in order to adjust the content of buffer solution and hence, the volume of the elastin network. The humidities were controlled by solutions of H_2SO_4 in water. To obtain results in a closed system, the force-length measurements were performed in paraffin.

b The binding of FAS or other substrates

The elasticity was measured at a fixed content of solvent, namely 1g/g elastin. Prior to incubation in FAS solution, force-length measurements were performed in buffer. Then, the strips were incubated in a FAS solution of $10g/dm^3$. After absorption, the swollen strips were removed from the solution and the volume was re-adjusted to the volume before absorption by placing the strips in a dessicator. To prevent absorption of water, the force-length measurements were performed in paraffin. The change in elasticity modulus resulting from FAS absorption was found by comparing the measurements before and after absorption.

c The damage of the elastin network resulting from FAS absorption.

Measurements were performed on strips, in which FAS were absorbed and subsequently desorbed. The data were compared with those obtained after 0.5 days of incubation in buffer (see *a*).

The procedure of the preparation of the strips with FAS was identical to that described under *a*, except that the strips were incubated in FAS or other substrate ($10\ g/dm^3$) instead of buffer solution and that after the incubation, the FAS were desorbed by immersion in water for maximally 6 days. After that the strips were placed in dessicators of various humidities. The measurements were carried out in paraffin.

As can be seen in equation 3.13, the cross-sectional area of the strip must be known to determine *E*. The value for the cross-sectional area is estimated as follows:

$$A_i = \frac{V}{L_i} \approx \frac{M}{\rho L_i} \qquad [3.17]$$

where M is the total mass of the strip and ρ the density. The density, in turn, is approximated by a linear combination of the contributions from dry elastin, water and FAS:

$$\frac{1}{\rho} = \frac{1}{\rho_{el}} X_{el} + \frac{1}{\rho_{H_2O}} X_{H_2O} + \frac{1}{\rho_{FAS}} X_{FAS} \qquad [3.18]$$

where X represents the mass fraction of the component indicated.
Because of the thickness variation along the length of the strip, the cross-sectional area, thus obtained, is a rather crude estimation. In order to determine reliable values for the modulus, we repeated the measurements, mentioned in *a* and *c*, at various volumes. In case *b* we compared data before and after absorption, where the volume and, hence, the cross-sectional area is kept constant. The change in modulus can then be calculated without making use of the value for the cross-sectional area.
A summary of the samples, thus investigated, is given in table 3.1.

Table 3.1 Samples subjected to static modulus measurements

	solution	number of measurements	incubation time (days)	absorbed amount (g/g elastin)
a	buffer	16 (various V)	0.5	-
		5 ,,	3	-
		10 ,,	7	
b	laurate	4 (constant V)	2	0.12 ± 0.03
		2 ,,	4	0.16 ± 0.03
	myristate	5 ,,	1	0.11 ± 0.03
		3 ,,	4	0.32 ± 0.03
	palmitate	3 ,,	1	0.12 ± 0.03
		4 ,,	2	0.20 ± 0.05
		1 ,,	4	0.40 ± 0.05
c	laurate	5 (various V)	2	0.13 ± 0.03
		7 ,,	6	0.16 ± 0.03
	myristate	3 ,,	2	0.20 ± 0.05
		6 ,,	6	0.40 ± 0.05

3.3.2 Dynamic modulus measurements

Dynamic elasticity moduli were determined with an Overload Dynamics (Zwick, type 142510/00) force meter, in which the strip was deformed at constant velocity. In figure 3.2 an illustration of the experimental set up is given.

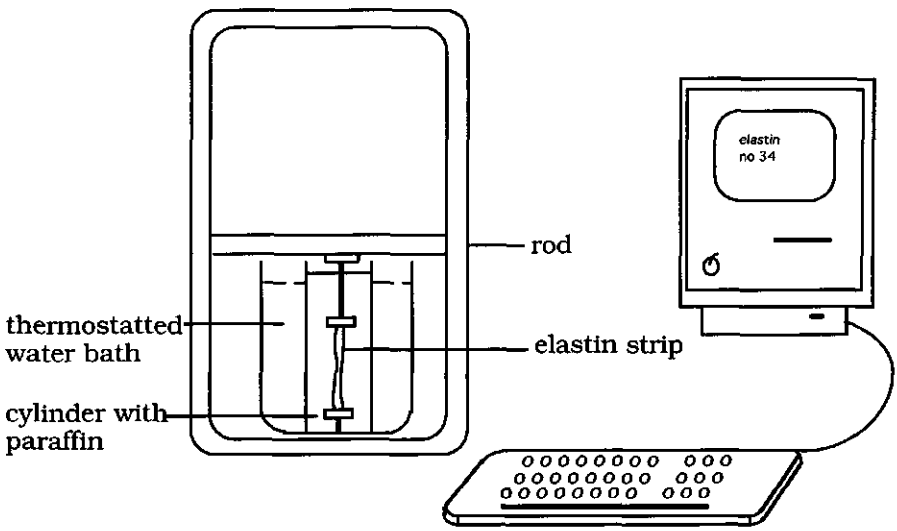

Figure 3.2 Schematic illustration of the Overload Dynamics

A strip with approximately the same length and cross-sectional area as used in the static modulus measurements was tightened between two clips and mounted into a cylinder, which was filled with paraffin. This cylinder was placed in a thermostatted water bath. The lowest clip was fixed at the bottom of the cylinder. The upper clip was attached to a sensor, which registered the force required to elongate the strip. The sensor was attached to a rod, which could move vertically.
A force of 0.2 N was exerted on the strip to have it slightly stretched in a vertical position and the length of the strip was measured. Then, the force was measured to stretch the strip by ca 2 mm. After this the force was released to bring the strip back to its original length, i.e. the length at 0.2 N. This cyclic deformation was carried out with a frequency of 0.1 Hz and repeated three times.

As in the static measurements the effect of the binding of FAS on the modulus of elastin was determined. However, for the dynamic measurements this was done at various temperatures, namely 37°C, 45°C and 65°C. The damage of the elastin network as a result of the binding of FAS was also determined. The preparations of the strips were similar as described under *a* and *b* for the static modulus measurements.

The preparation conditions of the samples for the dynamic measurements are summarised in table 3.2. At each incubation time, several strips having solution compositions within the ranges given in table 3.2, were measured. The remark '+desorption' in column 6 indicates that the measurements have been performed on strips with absorbed FAS *and* on the same strips from which FAS were desorbed.

Table 3.2 Samples subjected to dynamic modulus measurements

solutions		incubation time (days)	watercontent (g/g)	absorbed amount (g/g)	
37°C	buffer	1			
	laurate	2	0.5-2.5 *	0.12 ± 0.03	+desorption
		6	,, ,,	0.15 ± 0.03	+desorption
	myristate	2	0.5-1.5*	0.23 ± 0.05	+desorption
		6	,, ,,	0.37 ± 0.05	+desorption
	palmitate	1	0.5-1.0*	0.18 ± 0.05	
		3	,, ,,	0.34 ± 0.05	+desorption
		6	1.0-4.0*	0.60 ± 0.05	+desorption
45°C	buffer	5	1	-	
	laurate	5	2.	0.15 ± 0.03	
	myristate	5	3	0.35 ± 0.03	
	palmitate	5	3-4 *	0.45 ± 0.03	
65°C	laurate	5	1	0.15 ± 0.03	
	myristate	5	3-4 *	0.35 ± 0.03	
	palmitate	5	3-4 *	0.45 ± 0.03	

* water content varied within this range; for each strip the content was exactly known.

3.4 Results and discussion

3.4.1 Static modulus measurements

a Influence of incubation in buffer of pH = 10 at 70°C

By way of illustration figure 3.3 gives a typical result of the force-length measurements for elastin incubated in buffer for 0.5 days. It demonstrates that the length varies proportionally with the applied force. Such a linearity has been observed for strips under all conditions.

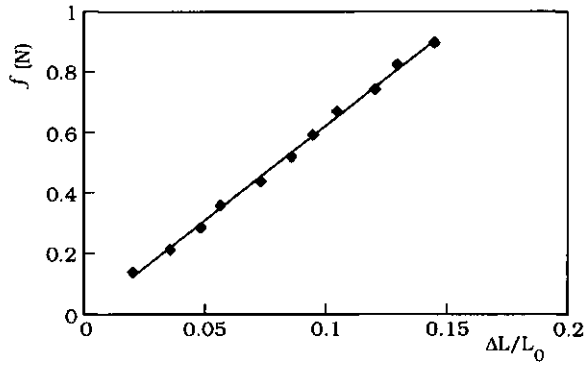

Figure 3.3
Example of a static modulus measurement of elastin in buffer at 70°C

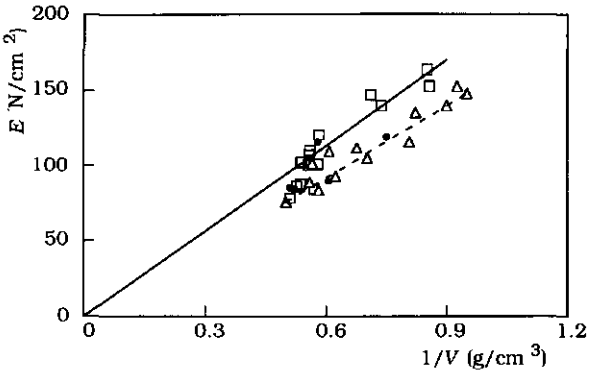

Figure 3.4
Modulus as a function of the reciprocal volume of a gram elastin in buffer at 70°C, determined with static measurements.
Measurements after
(□) 0.5
(●) 3 days
(Δ) 7days
Lines are drawn through data obtained after 0.5 and 7 days.

In figure 3.4 the elasticity modulus of elastin, which was incubated in buffer solution at 70°C, is plotted as a function of $1/V$. The full line is drawn through the data points for strips that are incubated for 0.5

days. The moduli for the strips, that were incubated for 7 days (dashed curves) are somewhat lower than those of the strips, incubated in buffer for 0.5 days. This points to some damage of the sample. According to [3.16] the slope of the line representing $E(1/V)$ is proportional to the number of cross-links ν . It is therefore concluded that this number slowly decreases during incubation in the buffer solution at 70°C. As judged from the decreased slope, after 7 days of incubation, the cross-link density is reduced by 17 ± 5%.

b Influence of binding of FAS

The modulus of elastin immersed in buffer for 0.5 days (1g buffer/g elastin) is 114 ± 20 N/cm^2. The large standard deviation (based on 16 samples) is mainly caused by the inhomogeneity of elastin and the uncertainty in the determination of the cross-sectional areas of the strips .

The modulus changes due to the absorption of FAS are collected in table 3.3. According to [3.14], the slope of the f($\Delta L/L_i$)-plot is EA. The measurements before and after absorption have been performed on the same strip and at constant volume, which implies that the cross-sectional area A is constant.

Table 3.3 Change in modulus of the elastin network as a function of incubation time in FAS solution.

	1 day	2 days	4 days
laurate		-5 ± 5%	-8 ± 6%
myristate	0 ± 1%		+10 ± 4%
palmitate	-2 ± 5%	-13 ± 6%	+12%

The data in table 3.3 indicate that the elasticity modulus slowly decreases during the first few days of incubation. It is noted that after 4 days an elasticity increase is observed in the case of myristate and palmitate. However, this result may be an artefact since after 4 days of incubation the samples did not behave fully elastically anymore, i.e. after releasing the force, the samples did not completely return to their original length. These strips were rather stiff because of the relatively high FAS uptake (0.3-0.4 g/g elastin) and low water content. In section

3.4.2 the viscoelastic characteristics of these samples will be further discussed.

c Damage of the network as a result of FAS uptake

The moduli of strips from which laurate and myristate were desorbed, after pre-absorption during 2 or 6 days, are shown in figures 3.5a and 3.5b, together with those for elastin incubated in buffer for 0.5 day. The fraction of ruptured cross-links, calculated from the slopes in figures 3.5a and b, are given in table 3.4 and compared with the cross-link rupture in elastin after 0.5 and 7 days of incubation in buffer.

It is clear that the presence of myristate causes irreversible damage to the elastin; the data suggest a similar but less pronounced effect for laurate.

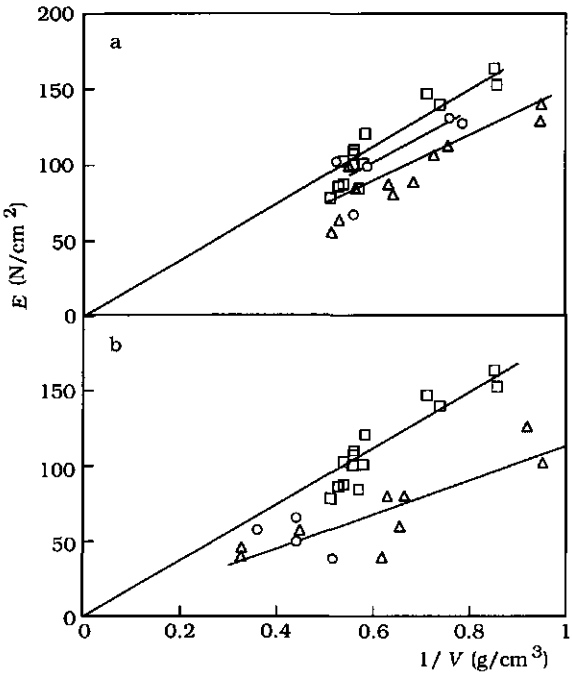

Figure 3.5 Static moduli of strips in which (a) laurate or (b) myristate was absorbed. Measurements after
(□) 0.5 day in buffer
(○) 2 days in FAS solution
(Δ) 6 days in FAS solution

Table 3.4 Change in cross-link density as derived from static measurements.

preparation of the strips	incubation time (days)	fraction of ruptured cross-links
buffer	0.5	0 %
buffer	7	17 ± 5 %
laurate (abs+des)	6	24 ± 6 %
myristate (abs+des)	6	39 ± 14 %

3.4.2 Dynamic modulus measurements

As compared to static modulus measurements, dynamic moduli provide more reliable information concerning the rheological properties of elastin.

As described in section 3.3.2, the force is measured as a function of length, which was changed with constant velocity. Figures 3.6 and 3.7 show examples of the output of such measurements. The plots in figure 3.6 are typical for a rubber-elastic material; the force as a function of length is identical for each cycle (fig.3.6a). Hence, the strip returns to its original position after releasing the stress (fig 3.6b).

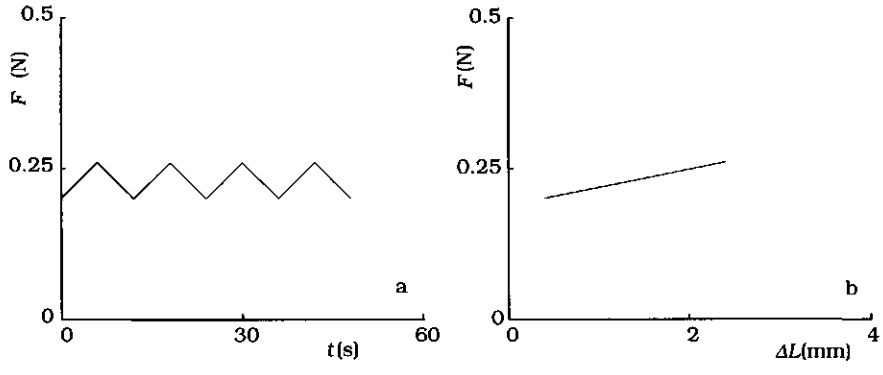

Figure 3.6 Dynamic measurements of rubber-elastic elastin.

In a viscoelastic material an irreversible change with time is superimposed on the cycles, as indicated in the figures 3.7a and 3.7b. Because the elastic behaviour of a material depends on the rate of deformation, the measurements are compared at fixed frequency, i.e. 0.1 Hz. The technique used in this study does not allow quantitative separation into the elastic and viscous component. Hence, the elasticity modulus of viscoelastic materials could not be determined.

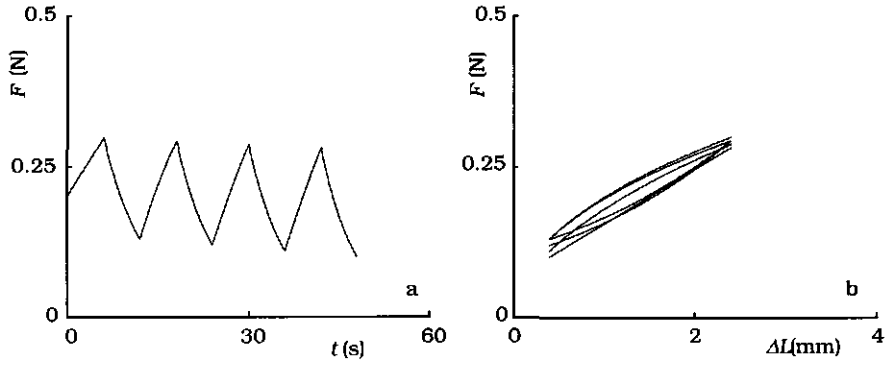

Figure 3.7 Dynamic measurements of viscoelastic elastin.

Rubber-elastic versus viscoelastic behaviour.

At 37°C elastin strips (without FAS) with water contents lower than 0.3g/g did not completely return to their original positions after releasing the stress. However, at higher water contents elastin was fully elastic under the same conditions. Lillie and Gosline[10] found that the transition from glassy into rubber elastic behaviour at one frequency depends on the water content and the temperature. They observed that the transition from the rubbery into the glassy state of elastin from aorta's sets in at a water content of 0.28g/g (at a frequency of 0.1Hz). Hence, in the case of elastin from bovine ligamentum nuchea, which is used in our experiments, it is plausible that the rubber-glass transition already occurred at a water content of 0.3 g/g elastin and 37°C, resulting in viscoelastic behaviour. In chapter 5 this transition will be discussed extensively.

None of the strips containing absorbed myristate and palmitate showed full rubber-elastic behaviour. Even at water contents above 0.4g/g elastin, the force-length plots were not completely reversible. However, laurate absorption did *not* result in an irreversible deformation, but the strips behaved rubber-like. The different behaviour for the various FAS is probably correlated to the differences in absorbed amounts; the strips with myristate and palmitate contained more FAS than those with laurate (table 3.2).
At 45°C, the rheological behaviour of the elastin strips is the same as that at 37°C, i.e. with myristate and palmitate they were viscoelastic, whereas with laurate they behaved rubber-like. However, at 65°C, the myristate and palmitate-containing elastin strips with a high water content (3-4g/g) were also completely reversible and hence rubber-like. In section 3.4.1 it was described that elastin with comparable amount of myristate and palmitate, but with smaller amount of water (1g/g) behaves viscoelastically. These results indicate that the rubber-like behaviour of elastin in the presence of FAS depends, as it does in its absence, on the temperature and water content [10]. However, to behave as a rubber at comparable temperature, elastin-FAS complexes require a higher water content than elastin. This is probable due to the larger number of polar groups in elastin-FAS complexes, which have to be hydrated in order to prevent inter-chain interaction and hence to suppress the viscous component in the modulus[11] .

Influence of FAS on the rubber-elasticity.
From the measurements on strips that behaved as a rubber the elasticity moduli E were determined. In figure 3.8a, the moduli of elastin with laurate, measured at 37°C, are compared with those of pure elastin, that was incubated in buffer for 1 day. It appeared that the moduli do not significantly change by absorption of laurate. However, figure 3.8b demonstrates that the modulus of pure elastin has decreased after absorption and subsequent desorption. It implies that the cross-link density did decrease due to the transient exposure to laurate. Combination of the results presented in the figures 3.8a and 3.8b suggests that the effect of cross-link density reduction on the elasticity modulus is compensated by an effect due to the mere presence of laurate in the elastin network.

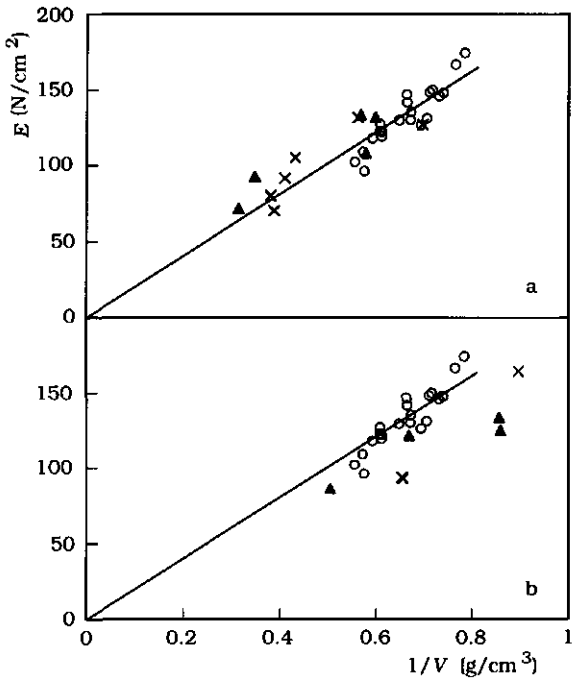

Figure 3.8
Dynamic moduli of elastin with absorbed laurate (a) and from which laurate was desorbed (b) at 37°C.
Measurements after
(O) 0.5 day in buffer.
(×) 2 days in lau- solution
(▲) 6 days in lau- solution
Lines are drawn though data obtained after 0.5 day in buffer

Measurements at 65°C show the same trend: i.e. the moduli of elastin, incubated in FAS solution for 5 days, do not significantly deviate from the line drawn through the moduli of elastin incubated in buffer for 0.5 days, (see figure 3.9), although due to the absorption, the cross-link density and, hence, the modulus of elastin itself, was decreased (see figure 3.5).

According to equation [3.16], the increase in modulus due to the mere presence of FAS can be explained by an increase in ν or a change in the constant C. If FAS forms cross-links, these would be non-covalent and break down readily upon applying even a weak force. Hence, such an increase in the number of weak cross-links, would not lead to a measurable increase in elasticity modulus. Anticipating the discussion in section 4.3, FAS absorption over the temperature range between 45°C and 80°C does not cause a significant variation in C. Extrapolating these data, it is probable that FAS absorption does neither affect C at T = 37°C.

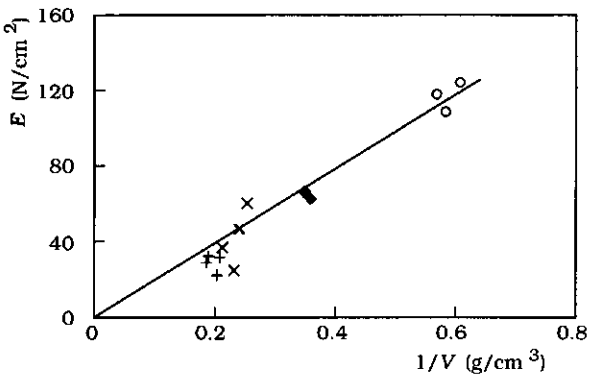

Figure 3.9 Dynamic moduli of elastin with absorbed FAS at 65°C. Measurements after 5 days of incubation in
(♦) laurate
(×) myristate
(+) palmitate
and
(○)1 day in buffer

The conclusion that neither an increase of ν nor C can be held responsible for the modulus increase leaves us with the question regarding the origin of the observed effect. It could be that the estimation of the cross-sectional area A_i underlying the validity of equation [3.18] is not sufficiently reliable. Furthermore, the fact that the elastin-FAS complex is near the transition from rubber- to viscoelastic behaviour complicates unambiguous interpretation of the experimental data.

Change in cross-link density

The influence of absorption and subsequent desorption of FAS on the cross-link density, as derived from the dynamic measurements, is given in table 3.5.

The modulus of elastin from which palmitate was desorbed after an incubation time of 6 days was also measured, but the result is not included in table 3.5. These elastin strips did not behave fully elastically. The most plausible explanation is that the cross-link density has reduced to such an extent that the network characteristics and, hence, the rubber-like properties, have faded away.

The data on the decrease in cross-link density of elastin after 6 days incubation in laurate and myristate (table 3.5) are in good agreement with those obtained from the static measurements (table 3.4).

Table 3.5 Change in cross-link density as derived from dynamic measurements

preparation of strips	incubation time (days)	fraction of broken cross-links
buffer	1	0 ± 1%
laurate (abs+des)	2	19 ± 10%
	6	21 ± 6%
myristate (abs+des)	2	15 ± 7%
	6	43 ± 8%
palmitate (abs+des)	2	9 ± 5%

3.5 Conclusions

The cross-link density, ν/V in elastin as a function of incubation time in FAS solutions, decreases as a function of time. The rate of degradation increases with increasing length of the hydrocarbon chain of the FAS. This result is in qualitative agreement with the conclusions concerning elastin degradation based on swelling (table 2.5, chapter 2). However, the rate of cross-link density reduction based on modulus measurements is higher than that derived from swelling. Because length-force measurements allow a more direct determination of the cross-link density, we consider the data derived from this method to be more reliable.

In addition to the influence of the FAS on the degradation of elastin, it may also change the rheological character of elastin. Depending on the water content, temperature and absorbed amount, FAS may induce a change from pure rubber elasticity into viscoelasticity.

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4

Conformational distribution of the polypeptide chains

4.1 Introduction

In a rubber network, randomly coiled chains are in rapid thermal motion. This rapid motion is possible only if large numbers of conformations are available to these chains. In general, not all conformations are equally compact. Depending on their rotational freedom, the chains may be found in a more compact or more extended conformation. The freedom of rotation depends on the rotational potential energy of the chain bonds, which, in turn, is affected by the size of neighbouring groups and the rotational freedom of neighbouring bonds. It may be expected that FAS absorption exerts a strong influence on the conformational distribution of the polypeptide chains in the network. In the present chapter this phenomenon will be investigated.

Under Θ conditions, there is a quantity $<r_0^2>$, which would be attained for a system in which excluded volume effects are absent. Although $<r_0^2>$ is obtained from force-length measurements when the chains are distorted, the values of $<r_0^2>$ refer to undistorted chains in solution. Thus, $<r_0^2>$ depends only on the conformational distribution of the chain elements. The value of $<r_0^2>$ is determined by the rotational freedom of the chain bonds, which is dependent on the rotational state of, at most, a few neighbouring bonds. Aaron and Gosline [1] found that in pure elastin, it takes approximately 7-8 amino acid residues per

chain element to exhibit the properties of a statistically, freely rotating chain. Usually, the nature of the diluent has a small effect on the rotational freedom of the chain bonds [2], and therefore, on the conformational distribution. If solvent molecules interact strongly with the chain, it is expected, and also found, that a conformational redistribution occurs [3,4]. In view of the strong FAS absorption in elastin, a redistribution, affecting $< r_0^2 >$, could be expected.
The quantity $< r_i^2 >$ is the mean square end-to-end distance of the network chains in the isotropic state.

4.2 Method

As has been mentioned in section 3.2.2, the expression for the retractive force, f, as a function of the elongation of a rubber, derived by Flory[5], reads:

$$f = \frac{\nu k T}{L_i} \frac{< r_i^2 >}{< r_0^2 >} \left(\alpha - \frac{1}{\alpha^2} \right) \qquad [4.1]$$

where ν is the number of chains (a chain is the polypeptide chain between two cross-links), k and T are Boltzmann's constant and the absolute temperature, respectively; L_i is the sample length in the isotropic state (i.e. in the absence of an applied force) and α is the extension ratio defined as L/L_i, where L is the length of the deformed strip.
Through motions around the chain bonds the polypeptide chains between two cross-links constantly change from one conformation into another. The average conformation of the network is represented by $< r_i^2 >$, the mean square end-to-end distance of the network chains in the isotropic state, and $< r_0^2 >$, the corresponding value for uncrosslinked chains in a Θ solvent [2,6].
At constant ν, L_i, $< r_i^2 >$ and α, f is proportional to $< r_0^2 >^{-1}$. Hence, force-length measurements carried out on elastin before and after absorption of FAS or, for a constant FAS-elastin composition, at different

temperatures, provide information about changes in $<r_0^2>$ and, consequently, changes in conformation.

$<r_0^2>$ before and after absorption

For rubbery samples the experimental curve for f versus α, invariably deviates from that described by equation 4.1, because the steric hindrance between the chains were not taken into account in the derivation of [4.1] [7,8]. Consequently, this equation is only applicable for elongations that are not too large. For natural rubber vulcanized with sulphur, experimental results were compared with the theoretical curve[9] based on equation 4.1. It appeared that the experiment fits the theory very well for elongations up to 75 %. Equation 4.1 holds better for swollen rubbers, because of the smaller steric hindrance[10].

For elastin, the following characteristic must also be taken into account. As has been found by others [11,12] as well as by us, for elastin from ligamentum nuchae the force depends linearly on the length. This behaviour is at variance with [4.1], which predicts df/dL to decrease with increasing length. The linear force-length curve can be explained by recalling that elastin consists of separate fibrils[11,12]. Therefore, when the network is extended to an extension ratio α, the α-values of the individual fibrils are different. At low extensions some of the more wavy fibrils do not contribute at all to the force, whereas they do at large deformations of the network[13]. The linear force-length behaviour might then be the result of coincidental compensation of the decrease in df/dL with L, derived from equation 4.1, by the increasing number of contributing fibrils. An argument that adds to this assumption is that for elastin from porcine aorta it is found that df/dL increases upon stretching[11]. In that case, the increasing number of contributing fibrils has a stronger effect than the decrease in df/dL, according to equation 4.1. Wolisch[14] observed by microscopy that the fibrils in elastin from aorta are extremely waved. If we measure the retractive force of elastin at fixed sample volume and extension ratio, the extension ratios of the individual fibrils are also fixed. Under these circumstances the proposed method for obtaining changes in $<r_0^2>$ through elasticity measurements is justified.

Unfortunately, the volume of the FAS containing elastin samples could not always be perfectly matched to that before FAS absorption. Hence,

these volume differences must be taken into account. We assume that, for small volume changes, the α-values of the individual fibrils are dependent on the α-values of the elastin network. In that case any variation in volume affects the force through the factor $\left(< r_i^2 > / L_i\right)$. In the absence of an applied force, elastin is isotropic, which implies that both L_i^3 and $< r_i^2 >^{3/2}$ are proportional to the sample volume. We obtain for the sample at constant α and T:

$$\frac{< r_0^2 >_{FAS}}{< r_0^2 >_{buffer}} = \frac{\left(L_i / f\right)_{FAS}}{\left(L_i / f\right)_{buffer}} \qquad [4.2]$$

As mentioned before, this analysis also assumes a constant value for ν, the number of cross-linked chains. However, in chapter 3 it has been shown that during the absorption of FAS, the number of cross-links decreases. Hence [4.2] must be corrected into:

$$\frac{< r_0^2 >_{FAS}}{< r_0^2 >_{buffer}} = \frac{\left(L_i / f\right)_{FAS}}{\left(L_i / f\right)_{buffer}} \frac{\nu_{FAS}}{\nu_{buffer}} \qquad [4.3]$$

We assume that cross-link reduction does not cause a change in conformational distribution, which is a good approximation if the decrease in cross-link density is not too large, i.e, if elastin continues to consist of an elastic network. The decrease in cross-link density can be determined by comparing elasticity measurements before absorption and after desorption, according to:

$$\frac{\left(L_i / f\right)_{desorb}}{\left(L_i / f\right)_{buffer}} = \frac{\nu_{buffer}}{\nu_{desorb}} \qquad [4.4]$$

$< r_0^2 >$ as a function of T

As stated before, $< r_0^2 >$ represents the average over all possible conformations. In an ideal rubber, these conformations all have the same energy, which implies that the conformational distribution is independent of the temperature. Our intention is to determine whether

FAS absorption influences the rubbery behaviour of elastin, by examining the influence of the temperature on $< r_0^2 >$.
At fixed volume it follows from equation 4.1:

$$\left(\frac{\partial \ln f}{\partial T}\right)_L = \frac{1}{T} - \frac{d \ln < r_0^2 >}{dT} \qquad [4.5]$$

For a variety of amorphous polymer samples, measured values of temperature coefficients have yielded values for $d \ln < r_0^2 > / dT$ [2]. In most of these experiments the volume was not fixed, because, as a result of thermal expansion, the volume of the sample increases. Corrections were then required for these volume changes. An alternative method is provided by using diluent mixtures in which the volume-temperature coefficient is zero[15]. In that case the thermal expansion is just compensated by the loss of diluent.
If the diluent affects the conformational distribution, this is reflected in the results obtained for $< r_0^2 >$. Usually, these effects are relatively minor. In the case of elastin equation 4.5 was applied to force-temperature measurements in water-ethylene glycol mixtures in which the thermal change in volume was compensated[12]: It appeared that $d \ln < r_0^2 > / dT$ was zero within experimental error. Later experimental work[16] for various one-component diluents have confirmed that $d \ln < r_0^2 > / dT$ is small (on the order of 0.3×10^{-3} deg^{-1}) This is strong evidence that the elastin conformations, just like conformations of other polypeptides[17] are not sensitive to changes in temperature and interactions with diluents. All of this is in line with the entropically determined elasticity.
However, in view of the strong preferential FAS absorption, conformational changes are more likely to occur and deviations from this ideal entropical behaviour may be expected. With this in mind, we carried out similar thermo-elastic measurements in FAS solutions.

Instead of measuring the force-temperature coefficient at constant length, as required by equation 4.5, we have measured the length as a function of the temperature at constant force. From the fact that, at constant volume, f is a function of L and T, we obtain from equation 4.5:

$$\frac{d\ln \langle r_0^2 \rangle}{dT} = \frac{1}{T} + \left(\frac{\partial \ln L}{\partial T}\right)_f \left(\frac{\partial \ln f}{\partial \ln L}\right)_T \qquad [4.6]$$

Since in the case of elastin at constant temperature the force depends linearly on the length, we may write 11,12):

$$f = c\left(L - L_i\right) \qquad [4.7]$$

where c is a constant. By combining [4.6] and [4.7], we obtain:

$$\frac{d\ln \langle r_0^2 \rangle}{dT} = \frac{1}{T} + \frac{\alpha}{\alpha - 1}\left(\frac{\partial \ln L}{\partial T}\right)_f \qquad [4.8]$$

Thus, if a diluent or diluent mixture is found in which the volume of the unstretched sample is independent of T, $dL_i/dT = 0$, and the value of $d\ln \langle r_0^2 \rangle / dT$ may be obtained by measuring the temperature dependence of the length at constant force. The main advantage of this method is that the experimental condition of constant force is more easily satisfied than the condition of constant length (as required by applying equation 4.5).

4.3 Experimental

$\langle r_0^2 \rangle$ before and after absorption

The force-length measurements at constant temperature (70°C) were performed using the experimental set-up shown in figure 3.1 of chapter 3. The experimental procedure was similar to that described in section 3.3.1 *b*. Measurements were carried out before and after absorption, and after desorption. After absorption (various incubation times), the FAS-containing elastin samples were transferred into water, in order to desorb the FAS. As judged from the weight, after one day all FAS were desorbed. The volume of the elastin strip was determined and compared with that before absorption. If the two volumes were not the same, the volume after desorption was readjusted to the initial volume by placing

the strip in a dessicator with a controlled humidity. A summary of the samples, thus obtained, is given in table 4.3.1.

Table 4.3.1 Samples of static modulus measurements

FAS	incubation time (days)	absorbed amount g/g elastin	number of measurements absorption	desorption
laurate	2	0.12 ± 0.04	4	4
	4	0.15 ± 0.03	6	4
	5	0.15 ± 0.03	2	2
myristate	1	0.11 ± 0.03	6	2
	4	0.32 ± 0.04	2	4
palmitate	1	0.13 ± 0.02	4	1
	2	0.25 ± 0.05	6	8

$< r_0^2 >$ as a function of T

Prior to the thermo-elasticity measurements, a diluent must be found, in which the volume of the unstretched sample is independent of the temperature. Elastin strips were incubated at 70°C in buffer or FAS solution of 10g/l. Using the experimental set up of figure 3.1 (chapter 3), the lengths of the samples in buffer-ethylene glycol mixtures, with ratios varying between 50:50 to 90:10 volume percent, were measured. In the cases of FAS containing elastin samples, 10g/l of corresponding FAS was added to the mixture, in order to prevent desorption by dilution during the measurement. To prevent curling, a small force of 0.02N was suspended from the strip.

Subsequently, the length-temperature measurements at constant force were performed in that mixture of buffer and ethylene-glycol, in which the length did not change with temperature. The force applied to the strip was 0.31 N. The length was measured at temperatures varying between 45°C and 85°C. Table 4.3.2 summarizes the samples that are used in the thermo-elasticity measurements.

Table 4.3.2 Samples measured as a function of temperature

FAS	incubatin time (days)	absorbed amount (g/g)	number of measurements
none	0.5	-	3
laurate	3	0.12	1
myristate	1	0.11	3
	3	0.25	2

4.4 Results

$< r_0^2 >$ before and after absorption

The results derived from the modulus measurements of elastin, before and during FAS absorption and after desorption are presented in table 4.4.1. The ratios given in this table are calculated at α = 1.10. The difference between the length of the strip before and after absorption was at most 3%.

Table 4.4.1 Results from modulus measurements before and after absorption and after FAS desorption. $\alpha = 1.10$

FAS	incubation time (days)	$\frac{(L_i / f)_{FAS}}{(L_i / f)_{buffer}}$	$\frac{v_{desorb}}{v_{buffer}}$	$\frac{< r_0^2 >_{FAS}}{< r_0^2 >_{buffer}}$
laurate	2	1.03 ± 0.07	0.90 ± 0.07	0.93 ± 0.13
	4	1.04 ± 0.07	0.82 ± 0.06	0.85 ± 0.12
	5	1.03 ± 0.05	0.92 ± 0.09	0.95 ± 0.13
myristate	1	1.04 ± 0.05	1.00 ± 0.20	1.04 ± 0.2
	4	visco-elastic*	0.73 ± 0.05	visco-elastic
palmitate	1	1.06 ± 0.08	0.97 ± 0.11	1.03 ± 0.19
	2	1.10 ± 0.09	0.87 ± 0.09	0.96 ± 0.18

As has been shown in the chapters 2 and 3, the cross-link density decreases by absorbing FAS. The data of column 4 confirm this trend, although they are subject to a large statistical error. According to equation 4.4, the decrease in the cross-link density due to FAS

absorption must be taken into account when determining $< r_0^2 >$. These data are obtained from equation 4.3, combining the data in columns 3 and 4.
The values for $< r_0^2 >_{FAS} / < r_0^2 >_{buffer}$ do not significantly deviate from unity, which implies that the presence of FAS does not influence the conformational distribution of the polypeptide chains. For elastin containing relatively large amounts of myristate no statement could be made, because there was a change from the rubber-elastic character into visco-elastic behaviour. In chapter 3 it was also shown that prolonged incubation (4 days) of elastin in other FAS solution could cause visco-elastic behaviour (depending on the water content). The appearance of a viscous component implies destruction of cross-links; therefore no inference could be made any more about the conformational properties of chain segments.

$< r_0^2 >$ as a function of T
Figures 4.1 a-d show length-temperature plots of elastin obtained in a mixture of buffer and ethylene-glycol. It demonstrates that the composition of the solution determines the $L_i(T)$ functionality. The curves in figure 4.1c deserve special attention; in the low temperature range the length (i.e. the volume) of elastin decreases with increasing temperature, whereas at higher temperature the length increases. This implies that the interaction between elastin and the solvent changes from exothermic to endothermic. Lillie and Gosline[18] also observed such a transition at a water-ethylene glycol mixture. They also found that at 37°C and 60°C, ethylene glycol does not disturb the rubber-elastic behaviour, whereas at lower temperature (5 and 20°C) it does. In view of this, we performed our thermo-elasticity measurements in buffer-ethylene glycol mixtures at temperatures beyond 37°C.
As can be observed in figure 4.1, the length of an elastin strip without FAS does not change in a mixture consisting of 90% buffer and 10% ethylene glycol in the temperature range from 40°C to 80°C. The athermal diluents for the FAS-elastin samples are given in table 4.4.2. These diluent mixtures contain FAS at a concentration of $10 g/dm^3$.

Table 4.4.2 Mixtures in which $dL_0 / dT = 0$ for elastin with and without FAS.

FAS	buffer: EG	Temperature range
-	90:10	40°C-80°C
laurate	80:20	45°C-80°C
myristate	80:20	50°C-80°C

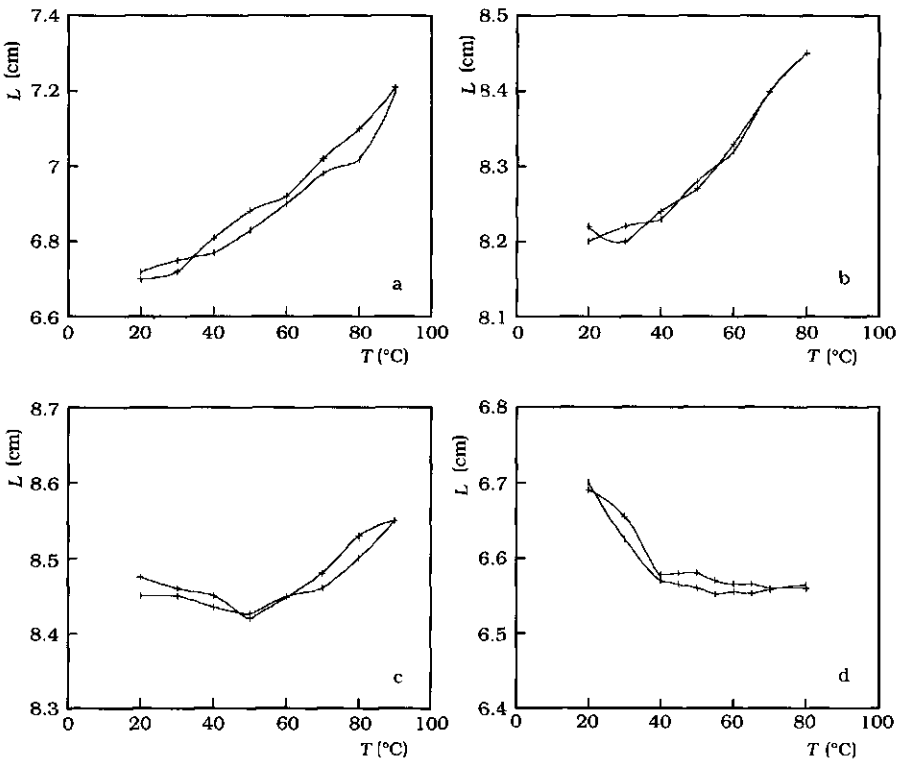

Figure 4.1
The change in length of elastin in the isotropic state as a function of temperature in various mixtures of water and ethylene-glycol. buffer : EG a) 60 : 40, b) 70 : 30, c) 80 : 20, d) 90 : 10

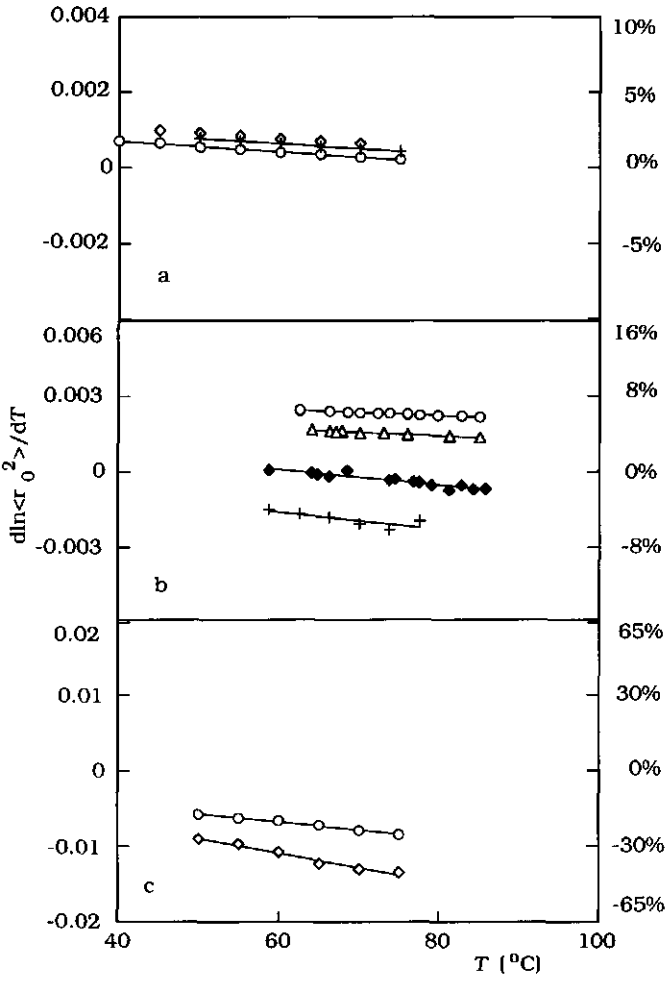

Figure 4.2
The change in conformation of
a) without FAS.
b)+ 0.15g/g laurate
Δ,○,◆ 0.15 g/g myristate
c) 0.4g/g myristate
Different symbols for the same system refer to repeated measurement.

Plots for $d\ln\langle r_0^2\rangle/dT$ are presented in figure 4.2. The values at the right hand axis represent the relative change in $\langle r_0^2\rangle$, when changing the temperature over an interval of 25°C. These values are calculated by integrating $d\ln\langle r_0^2\rangle/dT$ with respect to the temperature between 25°C and the value indicated on the abscissae axis.

For elastin without FAS, the variation in $< r_0^2 >$ is negligibly small, as it should be for an ideal rubber. The temperature dependence in the presence of FAS tends to be somewhat larger than that without FAS. However, the uncertainty in the determination of the length L relative to the temperature-induced variation in length L, gives rise to an error of approximately 15% in $< r_0^2 >_{T+25°C} / < r_0^2 >_T$. Hence, in view of the results shown in figure 4.2 a and b, the presence of 0.15 g laurate or myristate per gram elastin does not significantly influence the temperature-dependence of the conformational distribution of the polypeptide chains in elastin. In other words, laurate and myristate do not lead to significant deviations of the elastin network from rubber-like behaviour. However, as can be seen in figure 4.2c, larger amounts of myristate (0.4 g/g) do affect the temperature-dependence of the conformational distribution significantly, which implies that at such high FAS contents elastin does not behave as a rubber anymore. This result is in agreement with the conclusion in section '$< r_0^2 >$ *before and after absorption*' of this chapter.

4.5 Conclusion

In elastin-FAS complexes, the end-to-end length $< r_0^2 >$ of the polypeptide chains between two cross-links, does not change significantly because of FAS absorption, provided that the samples retain rubber-elastic behaviour. Hence, the rotational freedom of the polypeptide chain is not affected. It suggests that the FAS molecules do not bind directly to the polypeptide units in the main chain, but rather to the (hydrophobic) side groups. It is also possible that FAS molecules bind to the cross-links, either through hydrophobic interaction to the alkyl groups and/or through electrostatic interactions with the positively charged quarternairy ammonium groups.
At high FAS content the rheology of elastin changes from rubbery into visco-elastic. This supports the conclusion derived from static and dynamic elasticity modulus measurements, that FAS molecules if present in substantial amounts, do break down

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5

Glass-rubber transition

5.1 Introduction

In rubberlike materials, randomly coiled polymers are cross-linked into a network of chains, that are mobile around the chain bonds. Upon lowering the temperature, the number of accessible conformations around the chain bonds in the polymer chain decreases as a result of decreasing thermal energy and a decrease in volume, which results in crowding of neighbouring chains. Ultimately, over a relatively small temperature interval, rotational transitions become rare within the time of the measurement. In that case, the sample looses its rubber-elastic behaviour and a stiff, brittle structure results, the glassy state, with an elasticity modulus several decades higher than that of the rubbery state. The midpoint of this interval is usually denoted as the glass temperature or glass point T_g. Together with the change in elastic behaviour, the heat capacity decreases during the glass transition, whereas the specific volume remains almost constant[1]. The glass point is not a uniquely defined temperature, comparable to, for example the melting point, but its value depends on the time-scale of the measurement. The slower the lowering of the temperature, the lower is the value for T_g.

According to Kakiva and Hoeve[2], the glass transition of dry elastin occurs at approximately 200°C and decreases as the water content increases. In chapter 4 it has been amongst other things proposed that FAS may associate with the (non-polar) side groups of the amino acid residues of the polymer backbone, without affecting the distribution of

rotational states. It was concluded that FAS absorption has little or no influence on the entropy of chain segments between cross-links. Therefore, no influence of FAS on T_g is expected. In the present chapter this will be verified. To that end T_g is measured as a function of the water content in the absence and presence of FAS.

5.2 Method

Dry elastin samples of approximately 30 mg were immersed in FAS solution of 10g/dm^3. Subsequently, they were dried and the absorbed amount of FAS was determined gravimetrically. It varied between 0.2 and 0.4 g/g elastin. The glass transition temperature is measured as a function of the water content. To this end, the samples of pure elastin and elastin-FAS were placed in dessicators of various humidities for approximately 5 days. The humidities were controlled by various ratios of sulphuric acid and water. Then, the samples were enclosed in cups and the amount of water was established gravimetrically.
The glass-transition was determined by measuring the heat capacity, which was recorded using a Seteram Differential Scanning Calorimetry111 (DSC).
As mentioned in the introduction, the glass-transition point is not a uniquely defined temperature. It depends on the history of the sample. For example, in the glassy state relaxation to equilibrium is very slowly, and exothermic[3,4]. In order to compare samples with identical starting conditions, the samples were prepared by heating them at a rate of 4 K/min till the rubbery state is reached. Subsequently, the samples were cooled down at a rate of 5 K/min until ca. 50 K below T_g. Then, the glass transition was determined by again heating the samples with 4 K/min.

5.3 Results and discussion

Figure 5.1 represents, in a schematic manner, the heat capacity of elastin as a function of the temperature.

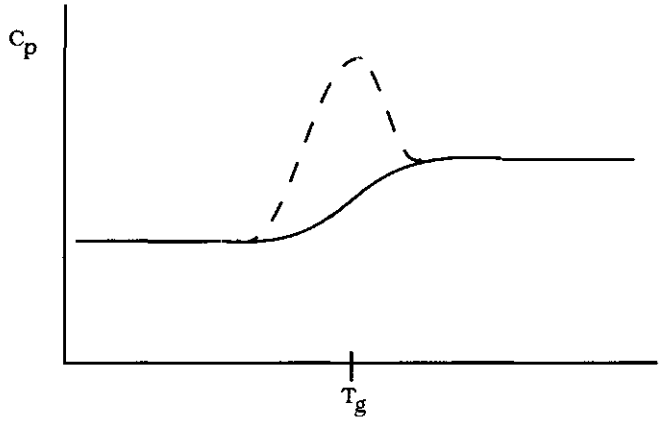

Figure 5.1
Schematic plot of the heat capacity as a function of temperature

The solid line is measured after elastin is heated to the rubbery state, cooled at 5 K/min and subsequently heated with 4 K/min (see method). The midpoint of the transition is denoted as the glass transition temperature T_g. For elastin, which was annealed for several days at a fixed temperature 20 to 30 degrees below T_g, the dashed curve was obtained, in which an endothermic peak is shown.

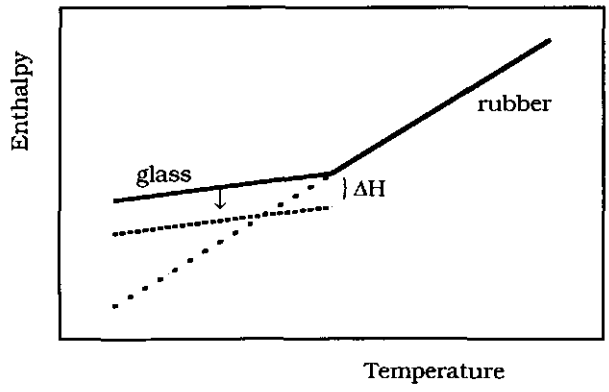

Figure 5.2
Schematic diagram of the enthalpy versus temperature. The arrow indicates annealing

Figure 5.2 shows a schematic presentation, explaining the appearence of this peak [3,4]. In the glassy state, the elastin relaxes very slowly, thereby lowering its enthalpy content. By heating the sample, after it has reached its relaxed glassy state, the glass transition will involve a positive enthalpy shift, which is reflected in a peak in the $C_p(T)$ curve. This peak will increase with increasing annealing period. In cases where

glass transitions were difficult to observe (e.g. elastin with high FAS content) , we made use of this phenomenon to establish T_g.

The amount of the various FAS absorbed in elastin, of which T_g. was determined are given in table 5.1.

Table 5.1 Elastin-FAS samples subjected to glass transition measurements.

FAS	g FAS /g elastin	number of samples
-	-	8
laurate	0.18 ± 0.02	7
myristate	0.30 ± 0.05	6
palmitate	0.22 ± 0.02	2
	0.08 ± 0.01	3
oleate	0.15 ± 0.04	4

Figure 5.3 shows the glass transition temperature of elastin and elastin with various FAS as a function of water content. The data for elastin without FAS resembles those measured by Kakiva and Hoeve[2]. The only difference being that they found a much larger dependence on the water content in the range between 0 and 0.05 gram water per gram elastin, resulting in a value for T_g of dry elastin which was approximately 50 K higher than ours. The glass transition decreases with increasing water content. This effect is not specific for water, since the lowering of T_g. was the same for equal volume fractions of water or ethylene glycol[5]. It suggests that the reduction in T_g is a non-specific diluent effect, i.e. T_g. decreases because at higher dilution content the polymer chains have less interactions amongst each other and, therefore, their rotational degrees of freedom are less restricted.
In chapter 4 we found that FAS does not change the conformational distribution of the polypeptide chains of elastin. From this we concluded that FAS does not interact with the polypeptide backbone, but rather with the non-polar side groups of the chains. The binding of FAS on the side groups may reduce the rotational degree of freedom of the polypeptide chain by steric hindrance. As can be seen in figure 5.3, the glass transition as a function of water content does hardly deviate

from that of pure elastin. Hence, binding of FAS only slightly, if at all, influences the rotational transition along the polypeptide chains.

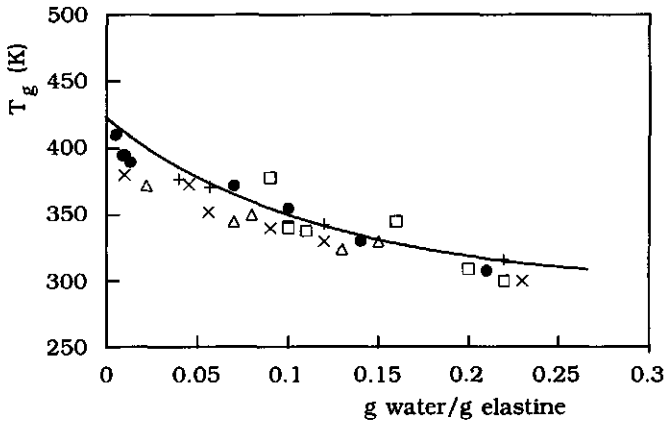

Figure 5.3
The Tg of elastin-FAS complexes as a function of water content. A curve is drawn through Tg of pure elastin. Measurements of elastin
(●) without FAS
(□) laurate
(×) myristate
(Δ) palmitate
(+) oleate

Differential scanning calorimetry scans of pure FAS, showed two endothermic phase transitions between 370 K and 410 K. It has been reported in literature[6,7,8,9] that for laurate the peak at 410 K is preceded by none, one or two transitions. The heat effect of the transition at 410 K in our study ($\Delta H \approx 7$kJ/mol) agreed very well with the heat effect, measured by Ferloni et al.[9] i.e. $\Delta H \approx 8$kJ/mol.
The transitions of the FAS also appeared in the DSC scans of our elastin-FAS samples and, surprisingly, the heat effects expressed per unit mass of FAS were as large as for pure FAS (7-9 kJ/mol). A high FAS content in elastin, sometimes obscures the glass-transition, because of overlapping by the phase transition of the FAS. The observation of the transitions of FAS that is absorbed in elastin implies that the FAS are not molecularly dispersed in the elastin network, but that they are present as aggregates. Hence, clusters of FAS occur at a few sites in the elastin. Thus, by far the largest part of elastin is not in contact with FAS. As a result, neither the glass transition nor $< r_0^2 >$ is directly influenced by FAS absorption. However, because FAS absorption results in swelling of elastin, with a concommittant increase of water, it indirectly lowers T_g.

5.4 Conclusions

Elastin undergoes a glass transition upon lowering the temperature. The transition temperature decreases upon increasing the water content of elastin, which can be explained by the increase of the number of accessible rotational states at higher water content.
The binding of FAS does not directly influence the glass transition, because FAS does not restrict the number of accessible rotational states. However, since absorption of FAS causes swelling of the elastin network, and therefore, increases the uptake of water, binding of FAS indirectly causes a shift in the glass temperature towards lower values.

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Summary

The aim of the present study was to investigate the interaction between salts of fatty acids (FAS) and elastin. Absorption of fatty acids in elastin may affect the elasticity of elastin-containing tissue. Such phenomena could, for instance, be of relevance for the understanding of the formation of atherosclerotic plaque in blood vessel walls.

Chapter 1 gives a general introduction on the relevance of this study and an outline of the thesis. Furthermore, it contains information on the characteristics of both elastin and FAS.

In chapter 2, experiments are discussed that give insight into the absorption mechanism. Elastin from bovine ligamentum nuchae was exposed to solutions of FAS having hydrocarbon chain lengths varying between 12 and 16 carbon atoms. The amount absorbed was determined gravimetrically. The uptake of FAS can be described by a second order mechanism, in which the absorption rate depends on the concentration of FAS in solution and the number of absorption sites in elastin, and in which the desorption depends on the number of occupied sites. Competitive absorption experiments showed that the absorption rate constant decreases with increasing chain length. The binding of FAS is reversible. Therefore it is allowed to calculate the standard Gibbs energy, $\Delta_{abs}G^0$ of the absorption process from the absorption isotherms. The affinity of the FAS for elastin is predominantly determined by hydrophobic interaction. The value for $\Delta_{abs}G^0$ per CH_2 group in the FAS is -0.8±0.2kJ/mol. This is much smaller than $\Delta_{abs}G^0$ for the transfer of CH_2 from an aqueous to a non-aqueous environment (-4kJ/mol). The difference may be explained by the fact that the FAS monomers that bind to elastin are released from micelles in solution. Although under the experimental condition of pH=10 where the experiment have been carried out, the overall electric charge in elastin is negative, the contribution from the negatively

charged head group of the FAS to $\Delta_{abs}G^0$ is attractive and equal to about -3kJ/mol. This suggests electrostatic attraction between FAS with positively charged groups, such as the desmosine and isodesmosine cross-links in elastin.
Binding of FAS is accompanied by co-absorption of salt (NaCl) and solvent in the elastin. The former results in an osmotic pressure between the elastin sample and the surrounding solution. This pressure increases as the salt concentration and/or the chain length of the FAS increases. The uptake of solution partly occurs to compensate for the electrostatic repulsion between the negatively charged groups of FAS in elastin. The swelling and, hence, the absorption of FAS is limited by the cross-linking of elastin. Due to the presence of OH^- ions and absorbed FAS, degradation of elastin occurs, resulting in a decrease in cross-link density which, in turn promotes the uptake of more FAS. The rate of cross-link rupture is proportional to the osmotic pressure, both are larger for FAS with a longer chain. The inference is that the degradation of elastin is enhanced by the internal osmotic pressure.

Elastin is a network of randomly coiled polypeptide chains displaying rubber-elastic properties. In chapters 3, 4 and 5 the influence of FAS on the rubber elasticity is studied.
In chapter 3, the change in elasticity modulus, which is the retractive force per unit cross-sectional area, is considered. The modulus depends on the number of cross-links per unit volume of elastin. Because of the degradation of elastin, caused by FAS uptake, the number of cross-links and hence, the elasticity modulus decrease. The degradation rate increases with increasing chain length of the absorbed FAS. In addition, FAS may also change the rheological character of elastin. After a relatively long incubation time, depending on the water content and temperature, FAS may induce a change from a pure rubber-like into viscoelastic behaviour.
The influence of FAS on the conformational distribution of the chains in the network is discussed in chapter 4. The end-to-end length of the polypeptide chains does not change significantly upon FAS absorption, provided that the samples retain their rubber-elastic character. In the case of viscoelastic behaviour, no inference about the conformational properties of the chains could be made. Furthermore, the dependency

of the conformational distribution on the temperature is hardly affected by FAS absorption. These results imply that the rotational freedom of the polypeptide chain is not significantly affected. The inference is made that the FAS molecules do not bind directly to the polypeptide units in the main chain, but rather to the (hydrophobic) side groups. It is also possible that FAS molecules bind to the cross-links, either through hydrophobic interaction with the alkyl groups and/or through electrostatic interactions with the positively charged quaternary ammonium group of the desmosine or isodesmosine units.

Upon lowering the temperature, the number of accessible conformations of the polypeptide chain decreases, which results in a rubber-glass transition over a relatively narrow temperature range. The transition temperature (T_g) decreases with increasing water content in elastin. This can be explained by the reduction of intermolecular interactions and therefore by an increase of the rotational freedom at higher water content. As it was concluded that the rotational freedom of the polypeptide chains is not affected by absorption of FAS, no influence of FAS on T_g is expected. In chapter 5, this expectation was confirmed. Binding of FAS does not directly influence the T_g. However, since absorption of FAS causes swelling of the elastin network, and, therefore increases the uptake of water, binding of FAS indirectly causes a shift of T_g towards lower values.

The most important conclusion concerning the interaction between FAS and elastin is that the direct influence of FAS on the elastic properties is very small. However, since FAS absorption is attended by swelling of the network and degradation of elastin, FAS absorption changes the rubber-elastic properties indirectly. The influence of the FAS is larger for longer chain length.

Samenvatting

Elastine komt voor in bindweefsel van o.a bloedvatwanden en de huid, en is verantwoordelijk voor de elastische eigenschappen van dit weefsel. Dit betekent dat een uitgerekt stuk elastine in zijn oorspronkelijke staat terugkeert als de kracht wordt weggenomen. Bij beschadiging van de bloedvatwand is het mogelijk dat het elastine in contact komt met het bloed en daaruit vetachtige stoffen zoals vet- en galzuren en cholesterol uit het bloed opneemt. Een vermindering van de elasticiteit van het elastine is het gevolg. De 'verslapping' van de ader kan de afzetting van plaque bevorderen. Dit proces zou een rol kunnen spelen bij de vaatwandziekte atherosclerose. Het is dus van biomedisch belang om de binding van vetachtige stoffen in elastine te bestuderen. In dit proefschrift is het onderzoek naar de absorptie van vetzuren en de invloed hiervan op de elastische eigenschappen beschreven. Hierbij is vooral aandacht besteed aan de invloed van de ketenlengte van de vetzuren.
Een beschrijving van de eigenschappen van elastine en de vetzuren is beschreven in hoofdstuk 1. Elastine bestaat uit polypeptide ketens die onderling verknoopt zijn. De ketens zijn niet gerangschikt in één of andere volgorde en zijn daarom wanordelijke polymeren. Omdat vetzuren niet goed oplosbaar zijn in water, is er gebruik gemaakt van de natriumzouten, in dit proefschrift afgekort tot FAS (fatty acid salts). De ketenlengte van de gebruikte vetzuren varieerde van 12 tot 18 koolstof atomen.

In hoofdstuk 2 worden verschillende experimenten besproken, die inzicht geven in het wisselwerkingsmechanisme tussen het elastine en de vetzuren. Stukjes elastine werden in FAS oplossingen geincubeerd. De opgenomen hoeveelheid werd bepaald door het drooggewicht van elastine voor en na de absorptie te vergelijken. Uit absorptie experimenten als functie van de tijd, bleek dat de opname van FAS beschreven kan worden volgens een tweede orde bindingsmechanisme,

waarbij de absorptie snelheid afhangt van de concentratie vetzuren in oplossing en het aantal lege absorptie plekken in het elastine. De desorptie snelheid hangt af van het aantal bezette plekken op het elastine. Experimenten, waarbij elastine is blootgesteld aan twee verschillende vetzuren hebben aangetoond dat de absorptie bij gelijke vetzuur-concentratie en gelijk aantal lege absorptie plekken langzamer gaat, naarmate de ketenlengte van het vetzuur toeneemt.

Vetzuren in oplossing vormen boven een bepaalde concentratie (CMC) micellen. Dit zijn klusters van FAS in oplossing, waarbij de ketens associeren en de negatieve geladen kopgroepen in contact staan met het oplosmiddel. De concentratie vetzuurmonomeren is ongeveer constant en gelijk aan de CMC. Boven deze concentratie bindt een vetzuur-monomeer aan het elastine. Tegelijkertijd gaat vanuit een micel, een ander monomeer in oplossing, zodat de concentratie vrije vetzuren constant blijft. De drijvende kracht achter de binding is het verschil in affiniteit van het FAS voor micellen en elastine.
Deze affiniteit is bepaald door de verandering in standaard Gibbs energieen, ΔG°, van het absorptie proces. Deze grootheid is te berekenen uit absorptie isothermen (metingen van de absorptie als functie van de concentratie FAS in evenwichts-toestand) Het bleek dat de affiniteit vooral bepaald wordt door de interactie van de keten van het FAS en deze neemt dan ook toe met toenemende ketenlengte. ΔG° per CH_2 groep is -0.8 kJ/mol. Dit is kleiner dan de ΔG° voor de verplaatsing van een CH_2 van een waterig naar een apolair oplosmiddel (-4kJ/mol), omdat de CH_2 vanuit het apolaire deel van het micel naar het netwerk gaat. De bijdrage van de negatief geladen kopgroep van het vetzuur is ook attractief en ΔG° is -3kJ/mol, ondanks het feit dat de nettolading van het elastine onder experimentele omstandigheden negatief is. Waarschijnlijk wordt dit veroorzaakt door de electrostatische wisselwerking tussen de FAS en positieve geladen groepen in het elastine, zoals die van knooppunten desmosine en isodesmosine.
De binding van FAS gaat gepaard met co-absorptie van zout en oplosmiddel. De absorptie van het zout zorgt voor een verschil in osmotische druk tussen de oplossing binnen en buiten het netwerk. Deze druk wordt groter naarmate de zoutconcentratie in de oplossing en/of de ketenlengte van het vetzuur groter wordt.

De opname van oplosmiddel is nodig om de electrostatische repulsie tussen de negatieve geladen groepen van de vetzuren (deels) te compenseren. De zwelling van het elastine netwerk en dus de opname van FAS wordt beperkt door de mate van verknoping van de polypeptide ketens. De knooppuntdichtheid wordt kleiner door de afbraak van elastine onder invloed van de OH^- ionen en geabsorbeerde FAS. Hierdoor kan de absorptie van vetzuren verder toenemen. De snelheid waarmee het aantal knooppunten verbroken wordt is evenredig met de osmotische druk. Beiden zijn groter naarmate de ketenlengte van het FAS groter wordt.

De ketens tussen de knooppunten in elastin bewegen snel. Dit is alleen mogelijk als een groot aantal mogelijke conformaties van de peptide eenheden in de ketens beschikbaar zijn. Bij uittrekken van elastine worden de ketens gestrekt. Als gevolg hiervan neemt het aantal mogelijke conformaties af. Dit heeft een verlaging van de entropie tot gevolg, waardoor de terugtrekkende kracht ontstaat. Dit is de meest kenmerkende eigenschap van een rubber. De invloed van vetzuren op de rubber-elastische eigenschappen van elastine zijn bestudeerd en beschreven in hoofdstuk 3,4 en 5.
In hoofdstuk 3 wordt de verandering van de elasticiteitsmodulus (een maat voor de elasticiteit van een stof) besproken. Deze modulus hangt af van de knooppuntdichtheid. Omdat binding van vetzuren afbraak van het elastine veroorzaakt, en dus de knooppunt-dichtheid vermindert, neemt de elasticiteitsmodulus af. De afname-snelheid van de elasticiteitsmodulus neemt toe met toenemende ketenlengte. FAS absorptie kan ook het reologische karakter van elastine veranderen. Na een lange incubatietijd in FAS oplossing kan het gedrag van elastine veranderen van puur rubberachtig naar visco-elastisch. De mate van verandering hangt echter ook van het watergehalte in elastine en de temperatuur af.
De invloed van de conformatieverdeling van de ketens is in hoofdstuk 4 besproken. Zolang het elastine zich als een rubber gedraagt, blijft deze verdeling het zelfde. In geval van viscoelastisch gedrag, kan geen conclusie getrokken worden omtrent de conformatieverandering.
In een rubber is de conformatieverdeling onafhankelijk van de temperatuur. FAS absorptie in elastine verandert deze temperatuur-

(on)afhankelijkheid nauwelijks. Deze resultaten duiden erop dat de rotatievrijheid van de peptide eenheden in de ketens niet significant verandert. Hieruit wordt geconcludeerd dat de FAS molekulen niet gebonden zijn aan de hoofdketen, maar eerder aan de (hydrofobe) zijketens. Het is ook mogelijk dat de FAS aan de knooppunten binden als gevolg van hydrofobe interacties met de alkylgroepen en/of door electrostatische wisselwerking met de positieve geladen groep van de quarternaire ammonium groep van desmosine en isodesmosine.

Bij verlaging van de temperatuur neemt het aantal mogelijke conformaties van een polypeptideketen af als gevolg van een verlaging van de thermische energie. Een overgang van rubber naar glas-achtig gedrag is het gevolg. Het midden van het temperatuurtraject waarin dit gebeurt is het glaspunt T_g. De invloed van water en FAS absorptie op het glaspunt is door middel van warmtecapaciteits (C_p) metingen bepaald en dit wordt besproken in hoofdstuk 5. De T_g van elastine is afhankelijk van het watergehalte, omdat dit de intermoleculaire wisselwerking in het netwerk vermindert. Hierdoor neemt de rotatievrijheid van de ketens toe. De T_g neemt daarom af bij toenemend watergehalte. De absorptie van FAS heeft, zoals verwacht, geen direkte invloed op T_g. De rotatievrijheid van de ketens wordt immers niet beinvloed door FAS absorptie (hoofdstuk 4). FAS beinvloed de T_g echter wel indirekt, omdat FAS absorptie gepaard gaat met zwelling van het netwerk, waardoor de T_g naar lagere temperatuur verschuift. Uit de C_p metingen bleek dat de vetzuren niet molekulair verdeeld, maar in klusters in het elastine voorkomen. Dit verklaart waarom de vetzuren weinig invloed op de rotatie vrijheid van de ketens heeft.

De belangrijkste conclusie omtrent de wisselwerking tussen vetzuren en elastine is dat FAS absorptie de rubber-elastische eigenschappen indirekt verandert door middel van het verbreken van knooppunten en de opname van oplosmiddel. De direkte invloed op de elastische eigenschappen zijn daarentegen erg klein.

Curriculum Vitae

Jannigje van Vreeswijk werd op 15 december 1964 geboren in Kamerik. In 1983 behaalde zij het diploma Atheneum B aan het Dr. F. H. de Bruyne Lyceum in Utrecht. In september van dat jaar ging zij scheikunde studeren aan de Rijksuniversiteit te Utrecht. De studie werd afgerond in februari 1989 met als hoofdvak Chemische Thermodynamica en keuzevakken Fysische Chemie, Chemische Informatica en Geschiedenis van de Natuurwetenschappen. Van april 1989 tot april 1994 was zij Assistent in Opleiding bij de vakgroep Fysische en Kolloidchemie aan de Landbouwuniversiteit in Wageningen. In deze periode werd het onderzoek uitgevoerd dat in dit proefschrift is beschreven.

Nawoord

Hoewel ik het uitvoeren van het onderzoek en het schrijven van dit proefschrift als een eenzaam avontuur heb ervaren, zijn er veel mensen geweest die mij op een of andere manier gesteund en/of geholpen hebben.

De direkte begeleiding was in handen van Willem Norde. Ik kon altijd bij hem binnen lopen om over het onderzoek te praten. Ook heb ik hem nooit horen mopperen als ik voor de vijfde of zesde keer een hoofdstuk voor correctie kwam brengen. Willem, het zal niet altijd makkelijk geweest zijn mij te begeleiden, want ik was nogal onvoorspelbaar. Bedankt voor je inzet en geduld.
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Tijdens het verzorgen van de colleges Algemene en Fysische Chemie was het in één groep erg gezellig. Later bleek dat er een fanclub voor mij was opgericht. Nog steeds geniet ik hiervan.

In het tweede jaar van mijn onderzoeksperiode ben ik de weg kwijtgeraakt. Al dolende liep ik tegen Josie aan, die mij weer op de goede weg zette. Het bleek een snelweg te zijn. Vanaf toen rolde Josie regelmatig met mij mee. Ik heb heel wat met haar gelachen, gehuild, serieuze gesprekken gevoerd en gek gedaan. Josie, ik zal ons dagelijks contact missen.
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Naast mijn collega's waren er ook andere mensen die op verschillende manieren een bijdrage aan de afgelopen periode hebben geleverd. Als ik al sudderend over een wetenschappelijk probleem thuis kwam, waren in het begin Marius en later René altijd bereid om mee te denken. Met mijn vriendinnen kon ik over van alles en nog wat praten en lachen.
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Promoveren dat doe je eens, maar nooit meer!

Yanet