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Contrast Intensification in the Electron Microscopy of Polymers

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CONTENTS

I. Introduction 298
II. Contrast in the electron microscope, and methods of increasing the contrast 298
III. Structural studies of polymers using contrast intensification methods. Effect of contrast materials 303

I. INTRODUCTION

The experience of recent years has shown that the properties of high-molecular-weight materials are determined essentially by their supermolecular structure. Therefore, information on the shape and size of regions of molecular aggregation of chains into bundle-like formations of different kinds is of interest. These structural features of polymeric materials can be investigated, mainly, by electron microscopy.

During the past decades, this method has produced a considerable volume of experimental data which has helped to correlate the properties of polymers with their structure. As a result, real improvements have been made in technological processes, but the problem is far from exhausted. The interlinking of amorphous and crystalline regions in polymers is still poorly understood. The mechanism of a number of transformations in polymeric materials (recrystallisation, polymorphic changes, etc.) has not yet been established. Further progress towards the clarification of these obscure areas of polymer science depends essentially on the development of high-resolution electron microscopy.

Currently available electron microscopes have a resolution of 4.5 Å, which allows crystal lattices with an interplanar spacing of ~ 2 Å to be photographed. However, the high resolving power of the electron microscope cannot always be exploited because of inadequate contrast in the image.

Image contrast in the electron microscope arises mainly from the scattering of electrons at the atoms of the object. Therefore, the contrast† of the electron-microscopic image is determined by properties of the object itself, such as density, thickness, and atomic number. If we fix the parameters of the electron microscope (aperture of the objective lens, accelerating potential) the contrast of the image increases in proportion to these properties of the object.

Organic materials do not meet the requirements for high contrast, because their molecules consist mainly of light atoms and their density usually lies in the range 1–2 g cm^{-3}.

Various methods, based on treating the object with substances containing heavy atoms of high scattering power, have been used to increase the contrast of electron-microscopic images of organic materials. These procedures have advanced most in the study of biological materials. Methods of contrast intensification for the electron microscopy of synthetic polymers are poorly developed, and have found only limited application.

An attempt has therefore been made, in the present article, briefly to review the contrast intensification procedures currently used for synthetic polymers and for biological materials. This is followed by a discussion of work on the morphological structure of polymers involving contrast enhancement by treatment with suitable materials.

Since contrast materials can modify the structure as well as reveal it, we shall consider the influence of contrast materials on the structure of an object being examined in the electron microscope. The review will begin with a short discussion of the dependence of image contrast on the parameters of the electron microscope and on the properties of the specimen.

II. CONTRAST IN THE ELECTRON MICROSCOPE AND METHODS OF INCREASING THE CONTRAST

Depending on the mechanism of image formation, we can distinguish between amplitude contrast and phase contrast in the electron microscope[1-3]. When an electron microscope is operated in the normal way without special attachments, phase contrast is produced by phase shifts arising from: (1) defocussing, and (2) spherical aberration. In addition, diffraction contrast due to the ordered arrangement of the atoms[4-10] can arise, in the study of crystalline materials, as a result of phase shifts.

Although phase shifts can increase the contrast to some extent, they add to the difficulty of a quantitative assessment of the image contrast because of their ambiguity.

At the present time, amplitude contrast plays the most important part in electron microscopy: it provides the basic information on the morphological structure of the object. Electron absorption makes an insignificant contribution and is ignored in practice[11-15].

Electrons which are scattered through angles greater than the aperture are absorbed by the diaphragm of the objective and do not take part in the formation of the image. Only those electrons which pass through the specimen unscattered, or scattered through small angles, contribute to image formation. Hence, the intensity of the electron beam is decreased after passage through the specimen. The intensity at the image of the specimen‡ can be expressed in the form

$$I = I_0 e^{-\frac{N_A}{A}\sigma_a \gamma x}, \qquad (1)$$

where N_A is Avogadro's number, A the atomic weight, γ the specific gravity of the object, x its thickness, I_0 the

† The term refers to the amplitude contrast of the image.

‡ The specimen is assumed to be amorphous.

intensity of the incident beam, and σ_α the effective cross-section of the atom. The contrast K of the electron microscope image is defined as the relative intensity of the electron beams I_1 and I_2 passing through neighbouring areas:

$$K = \frac{I_1 - I_2}{I_1} = 1 - \frac{I_2}{I_1}. \quad (2)$$

By combining Eqns. (1) and (2) we obtain an expression for the contrast K in terms of parameters of the object, in the form

$$K \simeq N_A \left(\frac{\sigma_{\alpha_2}}{A_2} \gamma_2 x_2 - \frac{\sigma_{\alpha_1}}{A_1} \gamma_1 x_1 \right). \quad (3)$$

This equation shows that contrast in the image arises as a result of differences in: (1) the scattering power of the atoms of the object, (2) the thickness, and (3) the density of the object.

The scattering power of an atom is characterised by the effective scattering cross-section σ of the atom. The effective cross-section of interest in electron microscopy is σ_α, which defines the probability of electrons being scattered at that atom by angles greater than the angular aperture α_0.[13,16-19] This is because the image is formed only by those electrons whose scatter angles lie within the angular aperture α_0 of the objective lens.

σ_α is a function of the atomic number Z, of the angular aperture α, and of the accelerating potential U. Since σ_α appears in the equation which defines the contrast, the latter depends on Z, α, and U as well as on γx; in other words, the contrast depends both on the observation conditions in the electron microscope and on the atomic number of the specimen.

It should be noted that the cross-section which appears in the contrast equation is the total cross-section $\sigma_\alpha = \sigma_{e\alpha} + \sigma_{u\alpha}$ (the sum of the effective cross-sections for elastic scattering, $\sigma_{e\alpha}$, and for inelastic scattering, $\sigma_{u\alpha}$, of electrons[20-22]).

1. Variation of Contrast with Accelerating Potential

A decrease of image contrast with increase of accelerating potential is a general feature of electron microscopy.

It has been stated[23,24] that the contrast is inversely proportional to the accelerating potential, or directly proportional to the square of the wavelength λ, since

$$\lambda = \frac{12.225}{\sqrt{U}} \text{ Å}.$$

However, a linear dependence of contrast on accelerating potential is only a special case, and can be realised only under certain observation conditions. In the more general example, the contrast is a power function of U, and therefore of λ^2.[24] The effective scattering cross-section which characterises the contrast is expressed in the form

$$\sigma_\alpha = A(\lambda^2)^l,$$

where A is a coefficient, l is an exponent depending on the particular apparatus, and λ is the wavelength.

Hence, it is desirable to lower the accelerating potential in order to improve the contrast. This problem has been discussed by many workers. Thus, Valentine[25] states that in microscopes with an accelerating potential of 5–6 kV the contrast is higher by a factor of 10 (as compared with 50–60 kV microscopes).

Some low-voltage electron microscopes have already been developed. Nixon[26] reports a considerable improvement in the image contrast by using an accelerating potential of 6 kV. However, the large gain in contrast offered by low-voltage electron microscopes cannot be fully exploited when the image is recorded on conventional photographic materials, because electrons of 6 kV energy cannot penetrate into the bulk of the emulsion, and this causes a loss of contrast in the photographic plate.

The use of electro-photography in low-voltage electron microscopes may enable the possibilities of the technique to be more fully realised. The first experiments in this direction, using electron beams of intermediate energies for electro-photography, have shown that the sensitivity of the method cannot compete with that of the more conventional methods of recording electron microscope images[27,28]. Nevertheless, low-voltage electron microscopes already offer improvements in contrast; these can be achieved by the use of various attachments to the electron microscope. In particular, the brightness intensifier not only allows the range of intensities of illumination of the object to be extended, but it also influences the contrast of the image. Existing methods of contrast intensification in electrophotography may also find application in electron microscopy, by using special photo-recording materials.

When organic materials are examined in a low-voltage electron microscope, allowance must be made for the increased thermal and radiation-chemical effect of the electron beam when the accelerating potential is decreased[29-34]. Thus, it is thought[23,35] that the optimum accelerating potentials for the electron microscopy of organic materials lie in the range 30–40 or 30–60 kV.

2. Variation of Contrast with the Angular Aperture of the Objective Lens

The general rule is that the contrast increases when the angular aperture of the objective lens is decreased. The variation of contrast with α_0 is strongly dependent on the atomic number Z of the material under examination[36-38]. In particular, it has been pointed out that the effective scattering cross-section σ_α (and therefore also the contrast) falls of rapidly with the angular aperture α_0 when Z is small. For large Z the decrease of σ_α with α_0 is more gradual.

For thin layers, and in the range of angular apertures $3 \times 10^{-4} < \alpha_0 < 1.7 \times 10^{-2}$, the relative change in contrast can be expressed in the form

$$\frac{K(\alpha)}{K(0)} = \frac{n_e}{n_e + n_u} \left[\frac{1}{1 + \left(\frac{\alpha_0^2}{\chi_0} \right)} + \frac{n_u}{n_e} \left(0.89 - 0.5 \ln \frac{\alpha_0}{3 \cdot 10^{-4}} \right) \right],$$

where $K(0)$ is the optimum contrast obtainable with $\alpha_0 > 0$; n_e and n_u are the numbers of elastic and inelastic collisions between electrons and the atoms of the object. Also,

$$\chi_0 = 0.84 Z^{1/3} \frac{\sqrt{1-\beta^2}}{\beta} \cdot 10^{-2}.$$

Fig. 1 shows the variation of the relative contrast $K(\alpha)/K(0)$ of the image of thin carbon films ($Z = 6$) with the aperture α_0 of the objective for two values of the accelerating potential, $U = 40$ kV and $U = 100$ kV.[17]

The aperture of the objective, α_0, is determined by the diameter of the aperture diaphragm of the objective lens, by the focal length of the objective, and also by the spherical aberration constant. Valentine has found that the contrast is doubled by changing the diaphragm diameter from

50 to 10 μm. However, extremely small diaphragm orifices rapidly become contaminated, giving rise to astigmatism. For this reason, Valentine recommends the use of diaphragm orifices 25 μm in diameter[23].

In Leisegang's book[17] changes in the accelerating potential and in the aperture of the objective are compared as methods for enhancing the contrast. The author considers it more satisfactory to increase the contrast by lowering the accelerating potential than the angular aperture. For a given object, he shows that the same contrast can be obtained at: (1) $U = 100$ kV, $\alpha_0 = 3 \times 10^{-4}$, and (2) $U = 40$ kV, $\alpha_0 = 10^{-2}$. However, under the former conditions the loss of resolution of the electron microscope is greater than in the latter.

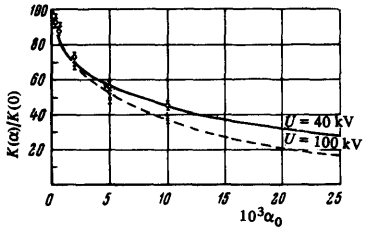


Figure 1. Relative contrast $K(\alpha)/K(0)$ of the image of thin carbon films against the aperture of the objective α_0 for two values of the accelerating potential[17].

Thus, the contrast of the image depends to a great extent on the conditions under which the object is observed. In order to obtain the maximum amount of information on the structure of the object with the help of an electron microscope, the optimum observation conditions should be provided. Pilyankevich has constructed a nomogram[42] which helps to choose the best experimental conditions.

3. Variation of Contrast with Atomic Number

As a general rule, the contrast increases with the atomic number Z[39-42].

The variation of the effective scattering cross-section σ_α with Z can be expressed in the form

$$\sigma_\alpha = AZ^k, \qquad (5)$$

where A and k are constants depending on α_0 and U. On increasing the angular aperture α, the variation of σ with Z tends to a quadratic form[3]. The linear variation of σ with Z is a special case, which applies only for particular values of the microscope parameters[17].

On this general rule is superimposed a periodic variation of the effective scattering cross-section with atomic number Z, which becomes less pronounced as the angular aperture of the objective increases[16,17]. Fig. 2 shows the periodic variation of σ with Z according to Pilyankevich[13]. Hence, not only the atomic number Z but also the position of the element in the periodic curve of σ_α against Z must be considered when calculating the contrast. It follows from Fig. 2 that the greatest contrast can be expected from substances such as iridium, osmium, rhenium, platinum, tungsten, gold, tantalum, chromium, uranium, and palladium, that is, elements for which the effective scattering cross-section lies on the peaks of the curve.

The amount of material needed to produce a standard contrast depends on the atomic number. Thus, the same contrast is obtained with a 50–70 Å layer of chromium, an 8–10 Å layer of gold, or a 3–7 Å layer of platinum or palladium[18].

4. Variation of Contrast with Density and Thickness of the Object

If the object consists of atoms of identical scattering power, image contrast can arise only as a result of differences in mass thickness (the product of thickness and density) of two adjacent areas[43-48]:

$$K = \frac{N_a \sigma_a}{A}(\gamma_2 x_2 - \gamma_1 x_1).$$

The molecules of most synthetic polymers and biological materials consist mainly of light atoms. Since N, C, and O atoms have practically the same scattering power, the

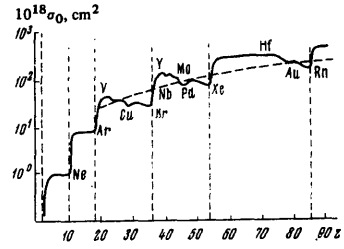


Figure 2. Variation of the effective scattering cross-section σ_0 with the atomic number Z of the elements[13].

image contrast of organic objects is due either to thickness variations or to density variations in the object being examined.

What is the thickness or density difference in an organic object needed to ensure the minimal contrast of the image which can be reliably recorded on photographic materials? (The minimal contrast for photographic materials is $K_{min} = 0.1$.) Valentine and Horne consider that if the product of thickness (in Å) and density (in g cm^{-3}) is greater than 400 the object will be distinctly visible, although a product greater than 100 is sufficient for the object to be detected on the photograph[49]. In the same paper it is shown that a photographic plate will not detect a difference in contrast for two materials of density 1.05 and 1.5 g cm^{-3} respectively: in other words, a density difference of 30% is insufficient to ensure appreciable image contrast.

Since density fluctuations in synthetic polymers and biological objects are usually much lower than this figure, the image contrast is affected almost exclusively by the thickness of the object.

The minimal thickness of an organic object which can be reliably recorded photographically has been discussed by many workers[46,47,50], who find that a thickness of at least 50 Å is necessary for satisfactory recording on a photographic plate. Elsewhere, figures of 100 Å are quoted. These values apply to electron microscopes of intermediate electron energies.

Pilyankevich and Stoyanova[24] investigated the minimal thickness Δd compatible with appreciable contrast as a

function of the accelerating potential of the electron microscope. They found that for $U = 50$ kV the minimal thickness is $\Delta d = 50$ Å (assuming $\rho = 1$ g cm^{-3}), whereas for $U = 200$ kV Δd increases to 500 Å. This means that the higher the accelerating potential, the greater must be the thickness of organic objects to be examined in the electron microscope. However, these results apply to the normal mode of operation of the electron microscope with image formation under bright field conditions. For dark-field images, the variation of the contrast with γ_x follows a different law, discussed in the work of Pilyankevich and Stoyanova. It is found that the dark-field offers a higher contrast than the bright-field method when the specimens are either very thin or very thick [51-54].

Thus, the potential use of electron microscopy in the study of organic materials meets with serious limitations associated with the contrast problem. Minor density changes in synthetic polymers and biological materials are not detected by the electron microscope. Hence, the contrast of organic objects reflects, essentially, only thickness variations in the material under study:

$$K = \frac{N_A \sigma_a}{A\gamma}(x_2 - x_1).$$

Consequently, materials which can be prepared as fine particles (more than 50 Å thick) are the most convenient for the electron microscopy of organic objects without contrast intensification.

In studies of ultra-thin sections having uniform thickness, the image contrast is practically the same in different parts of synthetic polymer or biological sections, giving no information on the morphological structure of these materials unless they are specially treated. Only rarely have differences in contrast of the sections been detected as a result of slight differences in thickness arising from specific features of the method of preparation (for example, in washing the section free from the mounting medium, etc.).

Similar difficulties, due to inadequate contrast, arise also with methods of preparation of organic objects other than the ultra-thin section method.

Various methods of treating the object with substances containing heavy atoms of high scattering power have been used to increase the image contrast of organic materials. The oldest and best developed method of contrast intensification is the shadowing method, which consists in depositing on the object a thin layer of metal, evaporated in a vacuum at a glancing angle to the object. The shadowing method was one of the first contrast intensification procedures, and is still widely used. It was first suggested by Williams and Wickoff [55] in 1944, and is now often applied in electron microscopic practice. However, this method allows one only to reveal the shape of the object and to reproduce the structure of its surface. Furthermore, in high-resolution work the structure of the evaporated layer itself becomes intrusive. The grain size of the evaporated material varies with the nature of the chosen metal and the method of shadowing used. A vast amount of literature has accumulated, both on the shadowing method itself and on its application to organic materials including biological specimens and synthetic polymers. The method is discussed in detail in monographs on electron microscopy [56,57].

Work on the method has been aimed mainly at finding a material and a shadowing technique to give a smaller grain size whilst retaining a high contrast. A relationship between grain size and the identity of the evaporated material has been demonstrated [58,59] in a study of a number of materials for shadowing electron microscopic objects. It is stated that the smallest grain size (down to 15 Å) can be obtained with platinum. The grain size can be brought down also by using special capillaries through which the metal vapour is directed at the object [60]. Noteworthy among these papers is that of Galyanov, who proposed a shadowing technique in which the metal is sputtered in a gas discharge, this allows the grain size to be decreased substantially [61]. Shadowing is used also when the object is examined by the replica method, as in the case of thick samples opaque to electrons. The commonest replica materials are quartz and carbon, both of which have a low scattering power. Hence, in order to increase the contrast, the replicas are shadowed with metals. In this method the quality of the replicas is determined essentially by their resolution. Bradley reports [62] that high-resolution replicas can be obtained by simultaneous evaporation of carbon and platinum. Marikhin obtained good results by evaporating quartz and platinum together [63]. Replica methods are described in detail in special articles and also in books on electron microscopy.

In the present article, however, we shall not dwell on this work, because we are concerned only with problems of contrast in direct examination, that is, in studies of the object itself rather than of its imprint.

Furthermore, we shall be concerned primarily with contrast intensification methods in which the object is impregnated with substances containing heavy atoms of high scattering power. These methods have been developed mainly in studies of biological objects, and have yielded more meaningful information on the morphological structure of these materials than has the shadowing method. In the field of synthetic polymers, the impregnation methods of contrast intensification are less well developed and have not been widely used. We shall therefore devote the major part of this article to those methods. Contrast intensification procedures which have been applied only to biological materials will be briefly mentioned.

Methods of contrast intensification involving impregnation of the object with materials containing heavy atoms were originally termed electronic staining, or simply "staining" methods, because of the obvious analogy with optical microscopy.

The term "staining" is now used mainly in the foreign literature, whereas "contrast intensification" is used in the Soviet literature.

The following methods of contrast intensification (or staining) in the study of organic materials by electron microscopy are known: (1) positive contrast intensification; (2) negative contrast intensification; (3) immunochemical contrast intensification; (4) the autoradiographic method; (5) the development of ordered regions; (6) the silver ion reduction method; and (7) decoration.

Positive contrast intensification or positive staining was one of the first methods for enhancing the contrast of organic objects [64,65]. Originally it was simply called "staining", but when a new method of contrast intensification (termed "negative contrast intensification") was developed, it became known as positive contrast intensification or positive staining. The method consists in impregnating the object with salts of heavy metals, and has been used with biological objects. In multicomponent structures, contrast is generated by the different amounts of metal which penetrate selectively into the structural elements of the object. Thus, osmium gives good contrast with lipoid structures, uranyl acetate with nuclei acids.

Negative contrast intensification (*negative staining* [69]) was first described towards the end

of the 1950's, and quickly became popular[66-70]. The principle of the method is that the particles being examined are surrounded with (or "*embedded*"[62] in) a strongly electron-scattering material, producing a contrast opposite to that obtained by positive staining. The negative staining method has been applied both to synthetic polymers and to biological polymers.

Immunochemical contrast intensification (*immunochemical staining*) is applied exclusively to biological objects[71]. The method depends on the binding of antibodies with antigens. Antigens (for example, virus antigens) become visible in the electron microscope if antibodies specific to the virus particles are previously chemically combined with proteins containing heavy atoms. Ferritin, which contains an iron atom, is frequently used as the protein. When the antibodies become bound to the antigens, the latter develop a higher contrast, which allows their location in the cell to be identified. The sensitivity of this method is extremely high.

The autoradiography method consists in treating the object with radioactive materials, which segregate at specific points[72,73]. A photosensitive layer similar to a photographic emulsion and containing silver atoms is then deposited on an ultra-thin section of the object. After exposure and photographic development of the object, silver particles are deposited on the parts of the cell where radioactive elements are present. The contrast of some of the cell structures is increased, so that they can be seen in the electron microscope. This method, also, is confined to biological objects.

The development of ordered regions is applied to oriented synthetic fibres. The object is impregnated with salts of heavy metals, which concentrate in the disordered regions of the fibre[74,75]. The object is pre-treated with reagents able to loosen the amorphous, disordered regions, thus assisting the penetration of the contrast-producing agent into those regions (but not into the ordered regions).

Contrast intensification by the reduction of silver ions has only recently been proposed. So far, it has been used and investigated only with graft polymers, guanine, poly(acrylic acid)[76], and DNA. The principle of this method is as follows. When the object has been treated with salts of heavy metals (for example, silver), silver crystals are deposited at the active groups of the polymer chain. The process is reminiscent of the formation of silver crystals during physical development. Since the silver nuclei have ~ 50 Å dimensions, we are able to see in the electron microscope the location of the specific groups of the polymer on which a hydrogen ion has been replaced by a silver ion, even though the silver atom itself does not meet the minimal requirements for contrast and is therefore invisible.

The decoration method has been adequately developed for inorganic crystals, in which it allows surface heterogeneities to be made evident[99,100]. The method is relatively simple, and consists in the high-vacuum evaporation under stated conditions, onto the surface of the object, of a metal (such as gold) whose particles can "label" the surface heterogeneities. This method has found practically no application in the study of organic materials. Only very recently has a preliminary report appeared of work by Bassett and coworkers on displaying the defects of the folded surface structure of polyethylene single crystals grown from solution.

Of all these methods, the negative contrast intensification method has attracted most attention in recent years for the electron microscopy of biological objects and synthetic polymers. A great deal of work has been published, particularly in the field of biology, using this method. Many workers have studied the method and have attempted to develop it further. Valentine and Horne[49], and Bradley[77] have reported some interesting studies of the negative contrast intensification method, of the materials used in it, and also of the resolution which can be expected from the method.

Somewhat conflicting views on the resolution of the negative contrast method have been published. Brenner and Horne[69] initially estimated the resolution as 15 Å. However, from recent microphotographic results, Bradley[77] obtains a resolution of 30 Å, reaching 15 Å only in special cases. Valentine and Horne quote figures of 20–30 Å[49]. Kay's book[57] states that details below 20 Å cannot be reliably investigated by negative contrast intensification.

Many workers have discussed the nature of the negative contrast intensification process, but a clear understanding of the interaction between the contrasting agent and the object is still lacking. Brenner, Valentine, Horne, Hall, and Bradley have all made interesting contributions in this area[43,49,50,69]. A paper by Müller and Meyerhoff[78] describes the "anomalous" contrast observed in negative contrast intensification: crystallites of MgO, deposited on a layer of tungstophosphoric acid, produce brighter areas in the image than the layer of contrast material. They ascribe this unusual effect to charges in the object itself. Later, Lippert studied the phenomenon in more detail by measuring the electrons scattered from the object and from the tungstophosphoric acid (TPA) during negative contrast intensification[79]. His experiments disprove Müller and Meyerhoff's hypothesis: Lippert considers that the "anomalous" contrast arises from the relative positions of the object and the TPA rather than from charges in the object. An MgO crystallite deposited on a layer of TPA is drawn into it, and scatters fewer electrons than the surrounding area of TPA. Thus, the "anomalous" contrast disappears, since the density distribution of scattered electrons in the image now varies in the same sense as the mass–thickness product of the object.

Phenomena leading to a loss of contrast also have to be considered in the electron microscopy of organic materials. In particular, a serious obstacle to the study of low-contrast objects is contamination by hydrocarbon vapours deposited on the object from the microscope column, which can lead to the formation of a thin film under the influence of the electron beam. A great deal of work has been done on this problem[80-84], but a discussion of this work would be out of place in the present review. The most recent designs of microscopes already include special attachments for minimising the rate of contamination of the specimen, and this undesirable phenomenon can now be readily overcome.

Thus, the contrast of the image of an organic object can be enhanced either by using the facilities offered by the electron microscope itself or by treating the object with materials of high scattering power. Since the optimum operating conditions in electron microscopic practice are usually chosen for fixed values of the apparatus parameters, most of the published work in this area deals with the problem of contrast intensification in the study of synthetic and biological polymers.

Published studies of biological objects and synthetic polymers by contrast intensification methods show that most of the effort is concentrated on revealing the fine structure of the specimens. The influence of the contrast agent on the specimen is attracting rather less attention, particularly in studies of biological polymers. However, the interaction between the contrast agent and the object is discussed in a number of papers. Therefore, we shall consider in detail not only the structural data obtained by electron microscopic studies of polymers but also the effect of the contrast-producing material on the objects being examined.

III. STRUCTURAL STUDIES OF POLYMERS USING CONTRAST INTENSIFICATION METHODS. EFFECT OF CONTRAST MATERIALS

Synthetic Polymers

The earliest electron microscopic studies of synthetic fibres using contrast intensification methods are due to Hess and his coworkers [74,75,85-89], who tried to detect large network spacings by electron microscopy. Large spacings had previously been observed in most synthetic polymer fibres by low-angle X-ray diffraction. Since the observed large spacings, of the order of 70–200 Å, did not match the length of any of the chemical groupings present in the polymers, it was suggested that superlattices appear in these materials as a result of the periodic alternation of ordered and disordered regions in the polymer. The sum of the lengths of these regions was identified with the observed large spacing. Large spacings were reported in fibres of polyethylene, poly(vinyl alcohol), and in polyamide fibres [89,90]. These materials were therefore investigated in the electron microscope with the aim of displaying the large spacing directly on the photographic plate.

It is well known that the density difference between amorphous and crystalline regions in polymers does not usually exceed 10%, which is not enough to provide the minimal contrast required in the electron microscopic image when working with electrons of intermediate energies, that is, under the normal experimental conditions. Therefore, the polymers were treated with materials containing heavy atoms of high scattering power, before being examined in the electron microscope, in order to enhance the contrast.

Essentially, these workers sought to increase the contrast by virtue of differences in the ability of amorphous and of crystalline regions of the polymers to sorb materials containing heavy atoms, thus producing enhanced contrast between the different regions of the specimen.

In an electron microscopic investigation of poly(vinyl alcohol) fibres, the specimens were treated with iodine after being steeped in formalin [74]. Large spacings of the order of 150 Å were revealed, as well as superspacings of the order of 700 Å. Examination of these fibres by X-ray diffraction confirmed a periodicity of 160 Å, but not the 700 Å superspacing.

Hess and coworkers used tungstophosphoric acid as the contrast enhancing material in their studies of polycaprolactam fibres [87]. The specimens were pretreated with a solution of HCl, which loosened the fibres and assisted the penetration of the contrast intensifier. These workers made a comparative study of the sorption of TPA in synthetic polymers and in collagen fibres. In the latter, the fine structure was developed by the chemical combination of a functional group of the protein molecule with TPA, which remained firmly held during the subsequent washing. In polycaprolactam fibres the TPA was sorbed only under special conditions (after swelling in a 9–10% solution of HCl).

The authors also studied the effect of HCl on the large spacing of polycaprolactam, and showed that the treatment produces a 30% shrinkage of the fibre length. Swelled fibres give more intense X-ray reflections, and their large spacing increases from 70 to 100 Å. After being pretreated with HCl solution, the fibres were dried and washed and treated with a 2% solution of TPA for 48 h. Under these conditions, sorption of TPA and penetration of contrast intensifier into the fibre takes place. The amount of TPA taken up was a function of the concentration of the HCl, that is, of the degree of swelling of the fibre.

High-angle X-ray diffraction studies of the treated fibres showed that the TPA is absorbed only by the disordered regions of the fibre, the crystallites remaining unaffected. The reflections also became stronger, indicating fewer defects in the crystal lattice and a higher degree of order. From the low-angle diffraction observations on treated fibres it was clear that the intensity of the meridian reflections increases with the concentration of the HCl. As the amount of TPA absorbed in the fibre increases, new reflections corresponding to 40–45 Å and 20–25 Å appear (the spacing is 70–90 Å at 9% absorption of TPA, 40–50 Å at 18% absorption).

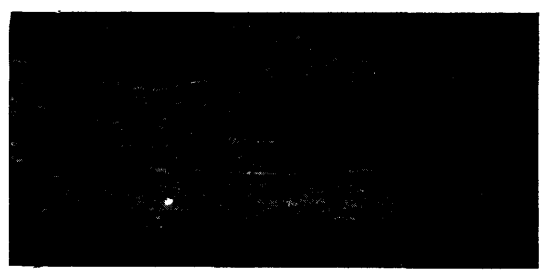


Figure 3. Polycaprolactam fibre stained with TPA [87].

Electron microscopic pictures of the dispersed fibres after treatment with HCl and TPA show that the fibre consists of thin fibrils 50–100 Å wide (Fig. 3). Along the fibrils, spacings of 70–90 Å can be distinguished as an alternation of light and dark bands. The authors point out that the periodicity along the fibre appeared only after treatment with HCl. The large spacing obtained from the X-ray diffraction work coincides with the value given by the electron micrographs.

Comparing the results of the electron microscopy of synthetic and natural fibres, Hess suggested that polycaprolactam is a better model of a protein fibre than poly(vinyl alcohol) or cellulose. This is because the material contains ϵ-aminohexanoic acid, which can react with the contrast intensifier TPA) to produce an electron microscopic image similar to that of collagens.

In 1958, a new method of contrast intensification was introduced into electron microscopy. The method, termed negative contrast intensification, has been widely applied in studies of biological objects. It has also been used by the present writers in studies of synthetic polymers [91-93]. Unlike Hess, who used TPA, we treated our specimens with

the potassium salt of TPA using the procedure suggested by Brenner and Horne[69] for biological objects. No pretreatment of any kind was given to the synthetic fibres. Contrast was achieved with both dispersed and ultra-thin sections of polycaprolactam, Lavsan [Dacron (Ed. of Translation)], and polyethylene fibres.

We had previously investigated the effect of TPA and its potassium salt on the structure of a number of synthetic fibres. The effect of the contrast agent was established by high-angle and low-angle X-ray diffraction, and also from electron microscopic and gravimetric data. From the normal high-angle diffraction patterns, the structure and the orientation of the crystallites in the fibres appeared to be unaffected by treatment with TPA or its potassium salt. Some changes were observed under low-angle diffraction. The intensity distribution curves obtained in an ionisation apparatus for treated and for untreated polycaprolactam fibres were used to calculate the large spacing and the size of the crystallites and of the amorphous regions[94]. The large spacing increased from 92 to 99 Å following treatment with 2 and 20% TPA respectively, the increase being due mainly to an increase in the size of the crystallites. The high-angle diffraction pattern of the fibre was unaffected by treatment in 20% TPA solution. An increase in weight not exceeding 5%, due to absorption of TPA, was observed by weighing the fibres after lengthy treatment in 20% TPA.

Electron micrographs of fibres which had not been washed after being treated with TPA showed extensive absorption of the staining agent, leading to a general increase in the level of intensity but making the structural elements indistinguishable.



Figure 4. Dispersed polyethylene fibre, stained with the potassium salt of TPA[94].

The elements of the fine structure of the fibres were revealed only by treating the dispersed fibres with the potassium salt of TPA. The photomicrographs of a polyethylene fibre (Fig. 4) treated in this way show a system of parallel fibrils, made visible no doubt by the penetration of the TPA salt into the regions between fibrils. The width of the fibrils was constant for different fibres (70–80 Å). A similar system of fibrils was observed in a section of a Lavsan [Dacron] fibre which had been treated with the potassium salt of TPA. It should be noted that the fibrillar picture of the longitudinal section gives an idea of the structure of the compact material of the whole fibre before disintegration by ultrasonic waves. From these results, we were able to conclude that the potassium salt of TPA has no effect on the value of the large spacing, it does not affect the crystallites, and it penetrates only into the interfibrillary spaces and the amorphous regions, in minute amounts. Nevertheless, these amounts of TPA are sufficient to reveal the fine structure of the polymers on the electron micrographs. It is pointed out that TPA (unlike its potassium salt) affects the large spacing, albeit by a small amount. Furthermore, TPA penetrates the fibre extensively, raising the level of the contrast but obscuring the fine structure.

For these reasons, we used the potassium salt of TPA as a contrast intensifier to reveal the large spacing in synthetic polymers. However, we met some difficulties: the minute amounts of contrast material sorbed by the amorphous regions were not always sufficient to provide the minimal contrast needed in the electron microscopic image. Hence, our observations of large spacings did not extend to all parts of the specimen, but were confined to those regions where the concentration of the potassium salt of TPA happened to be high enough, but not so high as to obscure the structure. A large spacing was observed in the micrographs of the following dispersed synthetic fibres: polycaprolactam, Lavsan [Dacron], polyethylene, and

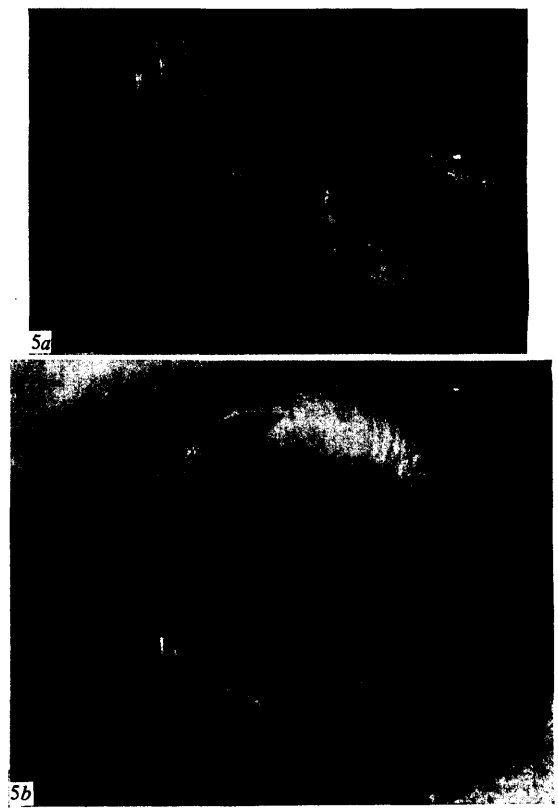


Figure 5. Negative contrast intensification of a polyester preparation[93]:
a) single crystals; *b*) spherulite.

pyrolysed polyethylene. The electron microscopic results agreed well with those obtained by X-ray diffraction. TPA was found to be an excellent contrast agent also for single crystals of polyesters. One of our papers[93] describes contrast intensification in polyester single crystals and spherulites. The latter clearly show a fibrillar structure with TPA contrast (Figs. 5*a* and 5*b*).

TPA was also used by Spit[95] as a contrast material for thin films of nylon, consisting of spherulitic structures with dark and light bands (revealed by the contrast treatment) of width equal to the known large spacing of the polymer under study.

Osmic acid (OsO_4) is often used as a contrast material in the electron microscopy of biological objects, because it has fixing as well as contrast properties. Marikhin and coworkers[96] investigated the contrast properties of OsO_4 on polymers by the low-angle X-ray diffraction method. They found that the intensity of low-angle reflections does not vary monotonically with the concentration of sorbates (iodine and OsO_4). The high-angle X-ray diffraction pictures show that these sorbates are concentrated in the amorphous regions of the polymers.

When multicomponent polymer systems are examined in the electron microscope, there are difficulties in revealing each individual component in the micrograph, because the polymers consist of light atoms having practically identical scattering powers. The inadequacy of the contrast raises special difficulties in work with ultra-thin sections of multicomponent structures, such as graft polymers. To distinguish between the different types of polymer in a graft fibre, a method of contrast intensification has been proposed for which we suggested the term "contrast intensification by reduction of silver ions"[76,97,98].

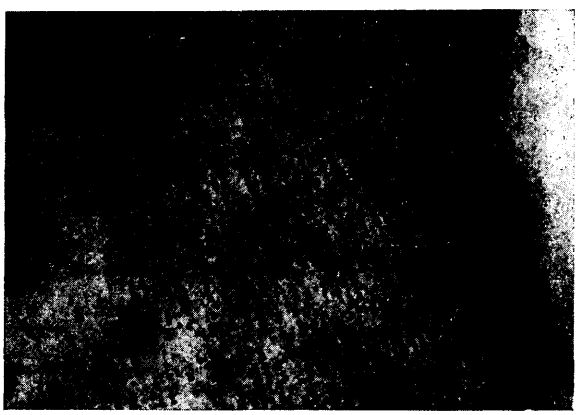


Figure 6. Cross-section of a polycaprolactam fibre treated with $AgNO_3$.[98]

The possibility of producing contrast in these polymers was demonstrated by the present writers by working with polycaprolactam fibres modified with poly(acrylic acid). The fibres were treated with salts of heavy metals: they were boiled for 3 h in saturated solutions of $AgNO_3$ and $HgNO_3$, causing partial replacement of carboxylate ions by heavy metal ions. By virtue of the powerful scattering effect on electrons of the Ag and Hg atoms, the location of the grafted material could be identified. An electron micrograph of the cross-section of a polycaprolactam fibre is reproduced in Fig. 6, where contrasting particles of 50–70 Å dimensions are clearly visible. The number of contrasting particles can be correlated with the percentage of grafted material, and decreases as the polymer being grafted penetrates into the bulk of the host polymer. In a polypropylene fibre with 5% of grafted material the number of particles is quite small. Dark-field observation of these particles showed them to be crystalline[98]. We have shown that the deposition of crystalline silver at the active groups of the polymer chain is not unlike the process of physical development in photography. Obviously, the first nuclei of the silver crystallites are formed at the poly(acrylic acid) sites, and this enables the disposition of the grafted polymer molecules to be seen in the electron micrograph. It was concluded from this work that the mechanism of this method of contrast intensification is similar to the mechanism of oxidation–reduction reactions.

Thus, contrast observation in ultra-thin sections can reveal the morphological structure of the object, but this cannot be done without special treatment of the object. In a few special instances, however, the fibril structure of fibre sections can be observed without artificial contrast thanks to differences in thickness between the different parts of the object, which can be observed after special preparative techniques[101].

Cellulose

The early electron microscopic studies of cellulose were made in the 1940's, before the preparation and staining techniques had been fully developed. The object was prepared by coarse mechanical comminution[102,103], and good preparations, transparent to electrons, were difficult to obtain. The specimens were in no way stained, and the image contrast was determined by differences in thickness only. Because of the low level of the preparative techniques, only a negligible amount of information on the structure of cellulose was gained from the electron microscopic results. The very first studies of cellulose by electron microscopy, reported by Ruska[102], allowed him to observe two layers in the fibre section: a compact outer layer consisting of fibrils, and a porous inner layer. Later work attempted to identify the structural units into which the fibres break down when disintegrated[103]. X-ray diffraction[104,105] was often used in this work to complement electron microscopy; electron diffraction[106–108] was sometimes employed. Fibrillar structures were invariably observed in electron microscopic studies of cellulose, irrespective of the method of preparation. The width of the microfibril, as measured on the electron micrograph, is invariably between 60 and 200 Å, depending on the object and on the method of preparation. Cellulose filaments of 60 Å dimensions have been termed micellar[109]. After the technique of shadowing the object by vacuum evaporation of heavy metals had been proposed, a large number of papers on the electron microscopy of cellulose appeared. The fibrillar structure of the fibres was much more clearly demonstrated in this later work[75,110]. A model of the cellulose fibre was constructed with the help of X-ray diffraction studies.

The model proposed by Hess and Kissig involves alternate ordered and amorphous regions in the fibre. The microfibrils observed in the electron microscope could not be reconciled with this model. Hence, the objective of electron microscopic studies became to identify the structure of the microfibrils—the first structural units of cellulose fibres.

However, problems of contrast arose immediately when the structure of the individual microfibril (20–60 Å thick) was investigated. Since the thickness of the cellulose microfibril is near the lower limit of the condition for minimal contrast, a search for methods of enhancing the

contrast of the object began. Shadowing could not solve the problem, because it can do no more than outline the shape of the object and the relief of its surface, and furthermore it obscures the internal structure. Also, the grain size of the shadowing material varies between 15 and 50 Å, which imposes a further limitation on the development of fine detail. The first attempts to introduce contrast intensification into the electron microscopy of cellulose are due to Hess and coworkers[74,111,112]. Their work on poly(vinyl alcohol) established the possibility of impregnating oriented polymers with iodine. Iodine treatment was applied by Hess also to cellulose. Elongated cellulose fibres were treated with potassium iodide solution, dispersed, and examined in the electron microscope. The principle of this method depends on the unequal absorption



Figure 7. Dispersed cellulose fibres:
a) stained with iodine[74]; *b*) negative contrast obtained with the potassium salt of TPA[113].

of the contrast producing agent by the different portions of the fibre. Contrast was obtained by impregnating the object with iodine and with thallium. The work was aimed mainly at the application of the method to cellulose fibres, that is, at finding the conditions which control the sorption of iodine. Iodine was sorbed by the fibre in amounts corresponding to 12% by weight of the fibre. At this level of absorption, the X-ray diffraction patterns of the iodised fibre were indistinguishable from those of cellulose. The authors showed that, when thallium atoms are used to increase the contrast, the absorption should not exceed 6–8% of the weight of the fibre in order not to disrupt the structure. Thus, Hess's work on the structure of iodine-treated cellulose deals at the same time with the problem of the effect of the contrast material on the object being examined.

The electron micrographs of cellulose fibres treated as above (Fig. 7*a*) show very clearly, along the microfibrils, alternating dark and light transverse bands with repeat distances of 60–200 Å (small spacings) and 500–800 Å (large spacings). The authors examined various types of cellulose (ramie, Fortisan [rayon]) and the pictures were always identical. The ordered regions were invariably unaffected by the sorption of iodine, as shown by high-angle X-ray diffraction.

On the basis of the electron microscopic and X-ray diffraction results, Hess put forward a model of the elementary microfibril of cellulose. Ordered and disordered regions alternate periodically along the cellulose microfibril, and are separated by sharp boundaries, so that the ordered and the disordered regions of all fibrils occur at the same level. The thickness of the microfibril was 200–300 Å, and the micellar filaments (the thinnest of fibrils) were 60 Å wide. The latter value corresponded to the narrowest microfibrils observed after dispersion of the fibres with ultrasonic energy.

The fibrillar structure of cellulose is very clearly revealed in the electron micrographs when the specimen has been treated with the potassium salt of tungstophosphoric acid (TPA). A paper by Belavtseva and Tsvankin[113] describes an investigation of the structure of cellulose treated with TPA and its potassium salt, using high- and low-angle X-ray diffraction as well as electron microscopy. Special attention was paid to the effect of TPA and its salt on the structure of cellulose. The electron microscopic work with dispersed cellulose fibres led to the conclusion that after treatment with a 2% solution of TPA the contrast of the fibre is greatly enhanced, but the individual microfibril cannot be resolved. Treatment of the specimen with the potassium salt of TPA by the negative contrast intensification technique revealed individual microfibrils 70 Å wide and 500–2000 Å in length (Fig. 7*b*). In the photograph, dark regions are visible between fibrils: these are the potassium salt of TPA, which is deposited in the form of a chain of globules. The chain of contrast material had the same width and length as the microfibrils.

The difference between the electron micrographs obtained after treatment with TPA and with its potassium salt respectively, and also the influence of these materials on the structure of cellulose, could be explained by interpreting the X-ray diffraction results.

The high-angle diffraction patterns of the specimens were unaffected by treatment with the potassium salt of TPA, whereas if the specimen had been treated with TPA itself a strong background of the gaseous type appeared on the diffraction patterns. The position and texture of the reflections remained unchanged. These facts suggest that TPA molecules can penetrate into the pores but not into the ordered regions.

As has been previously established, the low-angle scattering of untreated fibres of cellulose arises from diffraction at the elongated pores, oriented along the texture axis[114]. The length of these pores is much greater than their cross-section, and therefore the scattering is concentrated at the equator of the diffraction pattern. Treatment with TPA and its potassium salt yielded different low-angle diffraction patterns. Fibres treated with TPA showed strong equatorial scattering, but after treatment with the potassium salt of TPA the scattering was almost isotropic. Calculations show that the strong scattering in the low-angle diffraction picture after treatment with TPA arises from diffraction at regions having an average cross-section of 30–50 Å, distributed in the form of elongated cylinders 400 Å in length: in other words, the TPA here is lodged in the pores in the form of cylinders. On the other hand, low-angle diffraction from fibres treated with the potassium salt of TPA leads to the conclusion that the TPA is present in the fibre as globules of ellipsoidal shape with axes 60 and 80 Å long. Thus, whereas low-angle scattering in the original fibre was due to density differences between the fibre and the pores, which are minimal, after treatment with TPA the pores are filled with the reagent, giving rise to the observed strong scattering. The authors

showed that when the specimen is treated with TPA (but not with the potassium salt of TPA) the contrast-producing agent—TPA—is absorbed in large quantities. This follows from the electron microscopic and the diffraction results as well as from the gravimetric results. The large amount of TPA filling the pores of the fibre also produces a greatly enhanced contrast in the electron microscopic image of the fibre, but it makes the microfibrils invisible because their scattering power is much less than that of the contrast material. Hence, TPA "stains" the whole of the fibre without revealing its fine structure. The electron microscopic results and those obtained by X-ray diffraction show that when the fibre is washed in distilled water the original state of affairs (before treatment of the fibre) is restored. Only occasionally do crystallites of TPA remain stuck in the pores after washing, and these can easily be seen in electron micrographs obtained under dark-field conditions.



Figure 8. Ultra-thin section of cellulose, treated with silver hydrosol[118].

The ultra-thin section method has been increasingly applied in recent years, as an alternative to the dispersion method, in studies of cellulose fibres. In this method, image contrast is obtained as a result of differences in thickness between different areas of the section, and the fibrillar nature of the fibres has sometimes been revealed in this way[115-117]. Only a few papers have been published on the contrast intensification of ultra-thin cellulose sections. In a paper[117] on the treatment of cellulose fibres with OsO_4 and $AgNO_3$ solutions, it is shown that the dark particles in fibre sections are located on internal surfaces of the fibre.

Salyaev[118] studied sections of plant roots treated with Ag and Pt hydrosols. The electron micrographs of the root sections clearly show, in the thickness of the cell wall, silver particles filling submicroscopic spaces and thus decorating the interfibrillar spaces of the cell walls (Fig. 8).

In the present article we have discussed only the work aimed at contrast intensification in cellulose, and structural problems (as well as other electron microscopic investigations) have been ignored. For information on these topics the reader is referred to a monograph on the electron microscopy of cellulose[119] and to various other publications.

Collagen

Regular striations similar to those found in cellulose and in synthetic fibres have been observed also in a number of biological polymers (for example, collagen). The present review is concerned mainly with synthetic polymers, and therefore we shall deal, briefly, only with the work on collagen, because the electron microscopic results for this material are in keeping with those obtained by X-ray diffraction, and furthermore the electron micrographs show a remarkable similarity to those of oriented synthetic polymers.

The first X-ray diffraction studies of collagen fibres[122,123] revealed the presence of a periodicity of 830, 432, and 838 Å. In this work, attention was devoted to the effect of reprecipitation on the crystal structure of the fibres[121]. Bear[122] observed a periodicity of 640 Å in the low-angle diffraction pattern of air-dried collagen tissue. When these tissues were examined in the electron microscope after mechanical disintegration but without any artificial staining, transverse striations with a periodicity of 500–900 Å were observed. The contrast was evidently due to differences in thickness between the different regions of the fibre. The periodic striations in collagen fibres are easily visible even after shadowing, although they are not discernible in synthetic fibres shadowed with metals.



Figure 9. Collagen treated with TPA[124].

The spacing of 640 Å has been shown, by X-ray diffraction, to be common to all collagen fibres whatever their origin.

By applying contrast intensification to collagen fibres, the periodic striation has been more clearly revealed in the electron micrographs. Furthermore, the application of contrast has led to the discovery in collagen fibres not only of large spacings (Fig. 9), but also of sub-periods within the large period or spacing. As for synthetic and cellulose fibres, OsO_4 and TPA have been used as contrast materials for collagen fibres[124,125]. In that work, dispersed collagen fibres treated with OsO_4 or with a 2% solution of TPA were found in the electron microscope to consist of a thick network of fibrils with clearly developed striations. The repeat distance was approximately 700–1000 Å (large spacing), which was itself made up of a large number of bands

30 Å wide. Collagen fibres can be very effectively "stained" by contrast materials, even the small sub-periods becoming visible, whereas in synthetic fibres it is quite difficult to reveal even the large spacing.

Attempts have been made[126-129] to understand the chemistry of the interaction between TPA and collagen fibres. It has been suggested that polypeptide chains are responsible for the periodicity of the striations in the fibres: the TPA attaches itself to the amino-acid residues and therefore cannot be washed out with water. To confirm this hypothesis, photometric curves of the darkening of collagen fibres after contrast intensification with chromium and with TPA were presented: small bands (sub-periods) are observed only with preparations treated with TPA (not those treated with chromium). The small spacings (or sub-periods) in collagen fibres are thus ascribed to the presence of a chemical repeat distance along the fibre, such as would result from a regular alternation of atoms or groups, whereas no explanation could be put forward regarding the origin of the superperiods.

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The tendency of modern, quantitative electron microscopy is to exploit more fully the potentialities of the electron microscopic method. In particular, the image contrast is one of the limiting factors in quantitative electron microscopy. The papers presented at the Symposium on "Quantitative Investigations in Electron Microscopy"[130] discuss the possibilities and prospects in this area.

For this reason, recent publications have been concerned with problems of contrast and contrast intensification. In a number of instances, morphological and structural problems in crystalline polymers have been solved by diffraction methods[131]. However, polymeric materials of low scattering power usually need additional treatment aimed at increasing the contrast. We may conclude, from this review of work on contrast intensification in synthetic polymers, that the problem is still far from solved. Only the following methods for enhancing the contrast of a polymeric specimen are known: shadowing, enhancing the ordered regions, negative contrast intensification, decoration, and contrast intensification by the reduction of silver ions. However, with the exception of shadowing, these procedures have been applied to polymers on a very modest scale as compared with the extensive application of a variety of contrast methods in biology. This is because the scientific applications of electron microscopy have so far been mainly in the field of biology. Furthermore, the specific properties of biological objects are more compatible with the full exploitation of contrast intensification. With synthetic polymers, contrast intensification presents some real difficulties, and in most cases the choice of contrast agent must be made by *ad hoc* methods.

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