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PREFACE.

„. . . . . . *loin de pénétrer dans le domaine de la vie (le microscope électronique) ne plonge jusqu'ici que dans celui de la mort. C'est une contribution à cette thanatologie* *) *qui tente de plus en plus à se substituer à la biologie véritable*".

„. . . . . . *(Ces instruments) ne sont pas des microscopes, mais des* agrandisseurs. *Le microscope optique, le seul, le vrai microscope, n'est donc pas près d'être supplanté*".

M. Langeron, *Précis de Microscopie,* Paris 1949.

Pessimistic though the decisive verdict of the great French microscopist is, it did not prevent a crowd of scientists, *biologists* among them, from tearing themselves away from the familiar optical microscope to gather at Delft in July 1949, nor from rejecting, by the interest shown, the discouraging suggestion that the observation of animated things alone may reveal the secrets of Life. In short: The Conference on Electron Microscopy enjoyed a larger attendance than any member of the organizing committee had dared to hope. In particular the committee feels most honoured by the participation of so many distinguished scientists from abroad, some of whom had to travel a long way, and by the presence, at some of the meetings, of numerous non-electron microscopists who wanted to become acquainted with the latest achievements of the new instrument in the fields of biology, medicine and chemistry.

There has been a general demand for a book containing all the papers read at the Conference. Therefore we requested the authors to send us a copy and to include some of the micrographs shown. The cooperation we enjoyed was both enthusiastic and prolific. But now on producing this book we fear that some will hardly recognize their papers and even less their pictures. The predetermined price of the book compelled us to take red pencil and scissors. Moreover some alterations in the text seemed unavoidable and so we have to apologize to many.

The greater part of the participants gave ear to our request for passport photographs. These recall to memory the personal contact which is not the least important purpose of an international meeting. The excursion especially offered ample opportunity to make new friends. It also served the purpose of acquainting our guests with the most typical part of our country. In Amsterdam the municipal police gave a free demonstration of

*) thanatos (Gr.) = death.

our habit of eating raw carrots in the streets, besides protecting our car from the wrath of a green-grocer. Though we did not explicitly aim at living up to our reputation of wearing clogs and local costume, still everyone present at tea in Volendam returned home in the firm conviction that the small fishing town was the cradle of two well known electron microscopists.

On writing this preface we realize that, by the time it is printed, we shall already be halfway towards the next International Conference on Electron Microscopy. Should we call to mind that it was the French who — at Oxford — proposed to confer an official international character to these conferences? We look forward now to meeting again in Paris.

The editorial committee,

A. L. HOUWINK.
J. B. LE POOLE.
W. A. LE RÜTTE.

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188. A. Bengtsson, Fil. Kand., Fysikalisk-Kemiska Institutionen, Uppsala.
189. Prof. dr G. Glimstedt, Histologiska Institutionen, Lund.
190. A. Grudemo, Cement- och Betonginstitutet, Stockholm 26.
191. Ir E. de Haas, Allmänna Svenska Elektriska Aktiebolaget, Västerås.
192. C. G. Hedén, Avdelningen for Medicinsk Cellforskning, Karolinska Institutet, Stockholm 60.
193. G. Lagermalm, Svenska Textilforskningsinstitutet, Chalmers Tekniska Högskola, Göteborg.
194. B. R. Philip, Svenska Textilforskningsinstitutet, Chalmers Tekniska Högskola, Göteborg.
195. Dr E. Ribi, Fysikalisk-Kemiska Institutionen, Uppsala.
196. R. Rynninger, Svenska A. B. Philips, Gävlegatan 16, Stockholm.

197. Prof. dr F. Sjöstrand, Anatomiska Institutionen, Karolinska Institutet, Stockholm.
198. A. Syrrist, Tandläkarhögskolan, Malmo.
199. K. G. Thorsson, Avdelningen for Medicinsk Cellforskning, Karolinska Institutet, Stockholm 60.

SWITSERLAND:

200. Ir G. Induni, Trüb Täuber, Ampèrestrasze 10, Zürich.
201. Dr E. Kellenberger, Laboratoire de Microscopie électronique, Institut de Physique, Université, Genève.
202. Dr K. Mühlethaler, Eidg. Techn. Hochschule, Laboratorium für Elektronenmikroskopie, Universitätsstrasse 2, Zürich.
203. Dr H. Studer, Laboratorium für Elektronenmikroskopie, Anorganisch-Chemisches Institut der Universität, Freiestrasse 3, Bern.

TURKEY:

204. Prof. Fahir Yeniçai, Istanbul Üniversitesi, Fen Fakültesi Genel Fizik Enstitüsü, Istanbul.

U.S.A.:

205. Miss M. R. Ball, University of California, Los Angelos 24, Cal.
206. H. Halma, R.C.A., International Division, 745 Fifth Avenue, New York, N. Y.
207. Dr J. Hillier, R.C.A. Laboratories Division, Princeton, N. J.
208. Dr E. G. Ramberg, R.C.A. Laboratories Division, Princeton, N. J.

URUGUAY:

209. Prof. dr E. De Robertis, Instituto de Ciencias Biolôgicas, Avda Italia 3318, Montevideo.

OPENING ADDRESS TO THE CONFERENCE.

H. B. DORGELO, professor in
Physics of the Technical University at Delft.

It is my privilege to welcome you all, first on behalf of the Section for Applied Physics of the Netherlands Physical Society and secondly in the name of the members of the staff of the Laboratory for Technical Physics, especially those working there on electron microscopy.

We would like to extend a special welcome to those of you who came from abroad, from Belgium, Denmark, England, Finland, France, India, Norway, Portugal, Sweden, Switserland, Turkey and U.S.A.

We are grateful for the fact that the electron microscopy group of the Institute of Physics in England were ready to postpone their usual conference in favour of the conference here in Delft and we appreciate very much the help of Dr Cosslett from Cambridge in the organisation of this conference.

As is stated in the invitation to this conference, one of the primary purposes of the organisers is to encourage the direct exchange of views and experience between the scientific workers in the field of electron microscopy in different countries, and we are glad therefore to have here acknowledged pioneers in this subject and to welcome such a distinguished gathering of people who are interested in its problems.

There are many different aspects of the field of electron microscopy. Scientists of different kinds need each other to be able to attack the problems involved.

The aim of the scientist working on the construction and improvement of electron-microscopes is to procure for the users of the microscope the best possible tool.

The aim of the user of the microscope is to explore the unknown field of research into submicroscopic particles and to do that is fascinating and thrilling.
Electron microscopists, whether constructors or users of electron microscopes, have made and continue to make a significant contribution to science and technology.

The exchange of ideas between both categories of researchworkers is of the greatest importance for advances in both directions.

But there is more to say about that.

The electron microscope provides a meeting point for physicist, chemist and biologist. It is typical of science today, that there are many meeting points like that. I expect that out of such contact a new type of scientist will grow: the biophysicist.

Through the work of the constructors of electron microscopes the world has been enriched with an

instrument that has widened the field of research into submicroscopic particles of a size of 2000 down to about 15 A.U.

Compared with other methods for determining the size and shape of submicroscopic particles, such as the broadening of the lines in Debye-Scherrerdiagrams or the small angle scattering of X-rays by powders consisting of very small particles, research into submicroscopic particles with the electron microscope has unquestionably the advantage of being a more direct way of observing the size and the shape of these particles.

In the laboratory for Technical Physics here in Delft the three methods are now available and those of you who are interested in these methods can see and discuss them with the researchworkers in this field during your stay in our laboratory.

This is not the moment to go into a deeper discussion of the advantages and disadvantages of the different methods just mentioned for research on very small particles.

Allow me however to say a few words about the history of electron microscopy in Holland.

Before the second worldwar the foundation of electron microscopy was laid by Busch and the first commercial electron microscope was manufactured by Siemens.

In 1938 the Director of the Dutch Siemens Company was so kind as to give me an opportunity to see the Siemens electron microscope.

Not being able to obtain the money to buy such an instrument, yet being convinced that it would be of great importance for physical, chemical, biological, biochemical, biophysical and technical research, we decided to undertake the construction of such an apparatus in our laboratory.

The work of the team of the "Electron Microscope Institute" in our laboratory became possible by moral, intellectual, technical and financial help from many sides. I like to mention now especially the help of the Director of the Yeast-factory in Delft, Ir F. G. Waller, of Prof. Dr Ir A. J. Kluyver, the Director of the Laboratory for Microbiology, of the Council of the "Centrale Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (T.N.O.)" and the "Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.)" and of the Laboratory for Scientific Research of the Philips Works.

The work has been successful and we are thankful that this work and that of so many others has made it possible to hold the present conference.

We hope that this conference will stimulate research on and with the electron microscope not only through the formal lectures and discussions but also more informally during the excursions for example and by personal contact.

Expressing the hope that the atmosphere of this conference may stimulate creative thinking and promote personal friendship between those present, I declare this conference opened.

ELECTRON MICROSCOPES.

J. B. LE POOLE, T.P.D., E.M.Div., Delft.

The electron microscope is a typical example of an instrument which developed at an almost incredible rate. It is hardly 18 years now since the first transmission electron microscope was built and already we are coming together to-day in an effort to bring about, at the earliest possible moment the liquidation of a group among us, who may call themselves pioneers in this particular field. It is a well known fact that in many countries there were, and still are, societies for the study of the light microscope as such. To-day, however, the development of the light microscope has more or less come to a close owing to the progressed state of perfection this instrument had attained. The principles of image formation being well established, improvements now, important as some of them may be, are much like the last eruptions of a dying volcano. The light microscope has become a tool, and a powerful one.

This week a new instrument will be in discussion, which brought about an increase of resolving power of about 200 times. This is just as much as its predecessor, the optical microscope, did, compared to the naked eye.

In the realisation of the electron microscope the theory of the optical microscope was a great help indeed. Therefore it developed far quicker.

Still there are a number of specific differences. In fact, I guess that if Knoll and Ruska had known how many aberrations and artefacts exist in the electron microscope they would never have been stubborn enough to build one. The theoreticians at that time discussed the possibilities of using the extremely short wavelength of cathode-rays in combination with their ability to be focussed. Some of them came to the conclusion that an electron microscope could never be an improvement on the light microscope.

As I said, Knoll and Ruska were stubborn and apparently their electrons paid no heed to the solid articles and books.

Let us take now as starting point this first instrument built at the Technische Hochschule - Berlin. We will follow the electrons in their trajectories, and as we encounter the different components we will briefly discuss them and their later developments.

Of course, this first electron microscope was in principle a copy of the light microscope except for the details which were absolutely necessary to "translate" light optics into electron optics. In the first place there was the vacuum. The microscope therefore was pumped by a mercury diffusion pump provided

with a liquid air trap. The greater part of you nowadays prefer the oil-diffusion pump with its much greater speed, but I still think there is a lot to be said for the old mercury pump as it does not require the good backing vacuum. Moreover the liquid air trap greatly increases the pumping speed for vapours which often give rise to the notorious contamination. This effect, causing growth of particles, is almost certainly the result of decomposition of grease vapours by the cathode rays.

The electron gun was provided with a cold cathode which later on was changed for the well known V shaped filament, though the cold cathode has a few advantages especially for comparatively low voltages such as 50 kV. Those among you who had the privilege to give a large number of demonstrations will know the tendency of filaments to burn out at such occasions. There seems to be a marked relation between the importance of the persons demonstrated to and the probability of the burning out. Well, this of course cannot happen with cold cathodes. They just get a bit worse but don't stop working altogether.

On the other hand the hot cathode system suits the electron microscope better as the initial velocity of the electrons is smaller, a condition essential for extreme resolving power. Induni, however, who reintroduced the cold cathode in the Trüb-Täuber instrument, claims sufficient stability. According to him the initial velocities are of the order 10 Volts, which would indeed be small enough for even a very good resolution.

High tension was supplied by a normal 50 cycles transformer-rectifier unit, energised by a special generator, thus taking care of stabilisation. To reduce internal resistance really a collosal transformer was used. The smoothing condensors were also very big. In short a separate room was needed for the HT equipment.

Nowadays a 100 kV supply needs just about 2 or 3 cubic feet. Some of the commercial microscopes are equiped with high frequency supplies. thus putting into practice the suggestion which von Ardenne made in his book "Elektronenübermikroskopie". The HT supply of the R.C.A. microscope is claimed to be stable enough to measure the energy losses of the electrons in matter directly. That means that their energy after leaving a specimen can be measured by a β-spectrograph. The losses are computed by substraction of this energy from the initial energy. As the losses are small, a very high stability of the set-up is required. So the development of the electron microscope started to give by-products which are also of great value to modern science.

The specimen was brought in by means of an airlock. Recent developments in pumping technique made the usefulness of a specimen airlock in the modern electron microscope questionable as the whole instrument is pumped down in about 1,5 minutes. Still if a large number of specimens is to be investigated there is considerable difference between 15—20 seconds needed to restore high-vacuum after a good airlock has been in action, and 1,5 minute at the best to pump down the whole instrument. Besides it takes huge numbers of cigarettes to be waiting for high vacuum after the change.

To recap one might say: An airlock is useful provided it is well

designed. If it causes trouble the loss of time in looking after it soon outweighs the loss of time by not having an airlock.

The specimen itself is the most discussed point in the electron microscope. The study of organic material *e.g.* bacteria at a temperature of some 200° C seems very unpromising. Nobody could have foreseen that the electrons would have a fixing effect which might even prove to be better than any of the well established fixatives in microscopy, at least in this limited field. One of our own experiences may convince you. A biologist, about to complete an X-ray investigation of wax-needles formed by the epidermis of sugar-cane, finally wanted to know what they look like at high magnification. This looked very unpromising as the needles melt at about 450° C. Moreover the needles are comparatively thick *i.e.* 2 microns, so considerable energy exchange might take place. Experiments, however, showed that the needles did not melt. In fact we were not even able to melt them though we had occasionally molten Al_2O_3 by electron bombardment. (Melting point over 2000° C). The explanation of this most helpful fact may be that the electrons just lose enough energy to hydrogen atoms to knock them off the lattice.

No other atoms will be removed as the next lightest atom in organic material is carbon (atomic weight 12, so the transfer of energy is 12 times smaller), except at very high voltages. Perhaps this explains another fact: studying organic material with the high voltage microscope we occasionally found a very definite gain in penetration when increasing the voltage. After decreasing the voltage, however, much more internal structure remained visible than at the start. At first we thought that this was a psychological effect. Although it may be the effect of drying, it is also possible that some of the heavier atoms have been knocked out by the electron bombardment.

The exciting thing about these two phenomena is, that no apparent change of structure occurs.

So the old argument that it is impossible to study organic material in the electron microscope seems to be quite out of date. In fact people working with fixations for the light microscope give their preparations a much rougher treatment.

Both types of lenses, magnetic and electrostatic, have their specific qualities. A magnetic lens shows smaller errors, at least when used as objective, though the action of magnetic fields is far more complicated. Also for preliminary experiments the magnetic lens is the easier one to handle as it can be varied at will without extra controls. The greater the credit for those wo built electrostatic microscopes.

The most striking feature of the electrostatic lens is its constant focal distance. This is because its field is derived from the accelerating voltage. Therefore the HT supply needs not be as stable and can accordingly be simpler, although this does apparently not reduce the price.

Another unexpected thing about the electron microscope is that a number of lens errors hardly seem to matter. A few authors have even been explaining that some of the aberrations are helpful.

Perhaps it is good to remind you that contrast in the electron microscope is due to quite different phenomena than in the light microscope.

In the L.M. contrast results from differences in colour, in absorption, in scattering or refractive index. In the E.M. only scattering and refractive index or inner potential are responsible for contrast. Scattering is a comparatively simple phenomenon as long as amorphous matter is involved. In a decidedly regular lattice, however, scattering is very complex. It depends on a variety of quite incidental circumstances, *e.g.*, the position of the crystal lattice. Therefore contrast cannot, in this case, be used as a basis for calculation of thickness. As to the influence of inner potential on contrast: This is negligible as long as the object is in exact focus. Most of you will know the enormous gain in contrast by a slight reduction in power of the objective lens. This results from a phase contrast effect. This is another reason to distrust any calculation of thickness based on contrast.

To return to the small effect of lens errors: It is often said that spherical and chromatic aberration are favourable to contrast. The simplest way to obtain contrast in the electron microscope, from a theoretical point of view, is to introduce a physical aperture in the objective lens. The scattered electrons are caught on the diafragm and therefore play no part in the image formation. The more the scattering, the blacker the image. It is known that there is a good amount of contrast in the image without this aperture. The explanation is, that lens aberrations cause the most deflected electrons to hit the fluorescent screen at places outside the image, thus only giving rise to a background. Thus, instead of spoiling the image, lens errors take care of the contrast at least as far as spherical aberration and chromatic aberrations are concerned. So there is no reason why electrostatic lenses could not be as good as magnetic ones.

Is is clear now that we were fortunate to have a few pioneers who, in spite of the most pessimistic expectations and forecasts, built the instrument and made it work too.

For some time, *i.e.* a couple of years, the electron microscope was just a microscope. I would say it is rather a recent discovery that it possesses different properties from the light microscope. Electron beams are far more flexible than light rays, with all the advantages and disadvantages of that.

As to the disadvantages. If the inner wall of the microscope is charged, the electron beam will be deflected. If this charge is variable, the deflection will change too and the image will move.

Did you know that only a few millivolts asymmetric change in the potential of the inner wall is sufficient to give a detectable drift of the image? This means that changes of contact potentials owing *e.g.* to changes in the vacuum suffice to cause instability.

On the other hand, the flexibility of the electron beam may result in a great flexibility of the instrument. By means of a suitable design it is possible to have a quite useful, wide range of magnifications, adaption for other purposes, *e.g.* diffraction, oscillograms and nearly everything a sensible person might want. Actually the electron trajectories may be varied nearly at will, except for a few limitations by the properties of electrostatic and magnetic fields.

The projector lens in the first microscope was meant mainly to obtain the enormous final magnification. In the electrostatic microscope a twin lens was used to have two different magnifications. In the magnetic microscope changes in magnification were, and still are, made by changing the projector-current. Distortion in the projector may be corrected by special construction, and here the electrostatic lens has a definite advantage, because no rotational errors exist. So it seems a rather good combination to have a magnetic objective and an electrostatic projector.

To take *pictures* we have to consider the possibility of taking photographs of the fluorescent screen, as it would be a great help not to be compelled to bring photographic material into the vacuum.

Calculations and experiments, however, show that this means at least a loss of a factor 20 in exposure or 4,5 in definition or some combination in between. But what's more: There is a marked loss of contrast as the fast films needed for screen photography have very soft emulsions. Moreover the processing of the high sensitive materials involves working in complete darkness, which brings along more trouble than the use of slow emulsions for direct photography.

There remains the direct method of taking pictures. As the images are of a very low brightness the pupils of the eyes are at their widest. Therefore aberrations are considerable. To reduce the chromatic error of the eyes it would be an advantage to use a screen with almost monochromatic luminosity. Unfortunately this means the use of Willemite screens with a loss of 60 to 80 % in sensitivity. I think R.C.A. deserves the credit for having done special research and produced fluorescent materials for the electron microscope beating all others, not only in efficiency but also in contrast, at the same time having small grains. The phosphor, however, is a ZnCd sulfide, giving a rather wide range of wave-lengths, so maybe further improvement is still possible.

Anyhow there is a great difficulty in judging the quality of the image from the screen. So putting a photographic plate, which has a far better resolving power, underneath the screen, using the same or even greater magnification, seems to be a wrong solution. It has however, the advantage of being the simplest construction and if, by accident or by using special means as *e.g.* through-focus pictures, the image is of extreme quality, it stands a solid after-enlargement.

It seems rather logical to use the widely adopted 35 mm. technique also in electron microscopy. A number of advantages valid in normal photography also exist here. For instance: the use of less material per picture, resulting in smaller space required. The absolute necessity of using top quality enlargers, to take full advantage of the richness in detail of the image and the fact that the pictures are of no value whatsoever to the naked eye are disadvantages. Utmost care in enlarging and printing is required.

In the course of years several commercial microscopes appeared on the market. All of these have different shape and size and different features. They have, indeed, attained a certain state of perfection. This can be judged from the amount of attention, paid to minor details, usually called "selling points" *e.g.* chromium knobs, beautifully painted shieldings *etc.*

Now this was, of course, talking

nonsense about a most serious subject. Every one of the commercial instruments has its own merits. In every one of them the designers tried to express their hopes and believes. They all have established their great value in modern science.

A critical consideration of the pictures shows that the amount of detail is no longer limited by the electron microscope itself, but by the preparations. The next word has to be spoken by the users.

Meantime there is a wonderful task for the designers to adapt the electron microscope more and more to the requirements of easy maintenance.

Discussion.

Dr Ramberg remarks: Contamination arises from fixation of spreading surface films, not vapour fixation, as demonstrated by experiments by Dr Ellis. Observed also with mercury vapour pumps.

APPLICATIONS OF THE ELECTRON MICROSCOPE.

W. T. ASTBURY, Leeds.

Before proceeding to the more technical part of this short talk on the applications of electron microscopy, I should first like to make what is known in British parliamentary procedure as a 'personal statement'. It may be taken as read that I am proud to be asked to give this opening lecture, and my pleasure at meeting again my many Dutch friends is, I trust, perfectly obvious; but there is a special significance to me in this meeting at Delft, because it was here — in fact in the Laboratory of our President, Professor Dorgelo — that I first had the privilege of lecturing in Holland. That was in those far-off days when neither the electron microscope nor the war had yet been invented, but it is still one of my happiest memories.

And then again, in connection with Delft, there is something I feel we should all remember at a Conference such as this. The names of the great patriot, William the Silent, and of the great painter, Vermeer, automatically spring to mind when one thinks of Delft, but we of the breed of modern microscopists should recall too the name of Anthony van Leeuwenhoek, who also lived and worked in this beautiful old town. Van Leeuwenhoek was one of the Fathers of our subject, and we are not merely his scientific descendants, we are now actually re-living his adventures, though naturally on another level. Imagine his wonder and excitement, almost three hundred years ago, when first he peered through his single glass lens at the minute inhabitants of a drop of canal water, at the strange things in cheese, at even the pickings of his own teeth, and anything else that took his fancy! What a teeming, incredible underworld to come across, and what a thrill to be its first explorer! After three hundred years we are still exploring it, and whether we realise it or not, we electron microscopists are peculiarly fortunate: we are the first to look down with the human eye into the underworld of the molecules themselves. Here in Delft, once the scene of his pioneer discoveries and now a famous centre of electron microscopy, the keen, enquiring spirit of Anthony van Leeuwenhoek is surely looking down over our shoulders.

For my 'terms of reference' in this lecture the Conference Working Committee gave me one word only — "Applications", leaving me to define it, I presume, how I liked or as best I could. There is a wide-

spread story that compares an engineer (or it may be a journalist) with a physicist: the training of an engineer (or it may be a journalist) is to know less and less about more and more, while that of a physicist is to know more and more about less and less — you all know the story. Judged by this criterion all of us here fall under the description of physicists, since our aim — the great comprehensive "Application" of electron microscopy — is certainly to know more and more about less and less. It is not a bad definition, I think, so long as it is not stretched too far; for you will remember that the physicist in the story approaches asymptotically to a state in which he knows everything about nothing!

To illustrate this lecture I have of course invoked the help of colleagues in many laboratories, and they have responded so generously that I now find myself suffering from an embarrassment of electron microscopic riches. Electron microscopy is developing so rapidly and there are now available so many beautiful and instructive photographs from which to make a choice that it is no longer possible to do justice to them in a single short talk. You will understand, therefore, that perhaps ninety per cent of this lecture consists in reality of unspoken apologies. * It was a very difficult task indeed to decide what to leave out, and I will mention only the device I hit upon with regard to our Dutch friends. I argued to myself that I ought to leave them out altogether because, after all their trouble and hospitality in arranging this Conference, it would be quite ridiculous to waste their time showing them their own micrographs!

On the principle that the general aim of electron microscopy is to get to know more and more about less and less, the obvious way of classifying and describing the various "applications" is under headings prefixed by the word 'micro' — micro-this and micro-that, from microhistology in the upper reaches down to molecular studies in the lower. You will notice that already I am moving towards a predominantly biological viewpoint. That is not simply a personal bias, enthusiastic as I am about the structure of biological molecules and tissues; it is a fact that electron microscopy has so far found its most triumphant applications in the fields of biology, present results in other fields being — not exactly dull, but shall we say less informative and much less exciting. I sometimes joke at my orthodox histological friends by suggesting that their subject, at least as *they* practise it, is now obsolescent if not obsolete, and that they ought to consider seriously the question of resigning from their appointments. I would not wish to press very strongly the second half of this suggestion, but there is an element of truth in the first half. The electron microscope permits a so much more penetrating insight into the fine structure of biological tissues, and progress is now so fast and furious, that the time is by no means far off when revolutionary new text-books of histology will have to be written. Perhaps it may fall to the lot of one or more of us here today to shoulder this important task.

* This published summary is of necessity even more apologetic than the spoken word, for only a fraction of the pictures used to illustrate the actual lecture are reproduced here.

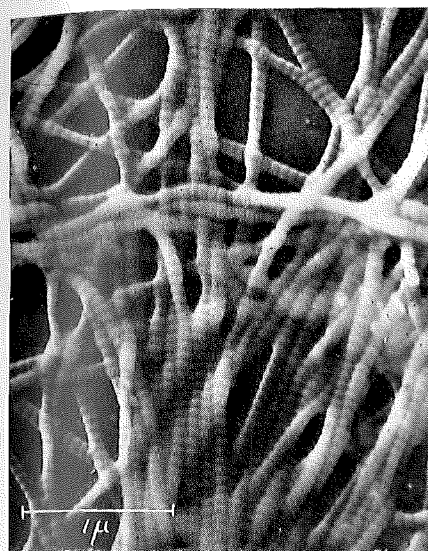


Fig. 1. Dermal fibers from flexor unexposed surface of fore arm of senile purpura patient (age 59 years). Cr-shadowed. (Reed, Tattersall and Millard).

We may choose, out of numerous possibilities, Figs. 1 and 4 to illustrate micro-histology. Fig. 1, a micrograph of collagen fibres in skin, was taken in our own laboratory at Leeds but otherwise makes no claim to be unique. The excuse for showing it here is partly to economise in the illustrations to the subsequent paper by Reed, Tattersall and Millard, and partly on the principle (see also Fig. 3) that "whosoever bloweth not his own trumpet, the same shall not be blown!" Quite a number of people have made electron microscope studies of collagenous and related structures — they constitute one of the most fascinating and instructive fields so far revealed — and the outstanding work of F. O. Schmitt and his collaborators, and of Wolpers, for example, is known to everyone. Similar remarks apply to the fine structure of muscle, illustrated by Fig. 4, one of several beautiful micrographs kindly sent to me from Australia. The pioneer photographs of this kind were taken by Schmitt, Hall and Jakus, who thereby made the fundamental observation that down to the limits of resolution of the electron microscope there is no visible folding or crumpling of the myofibrils during muscular contraction.

Micro-histology (or micro-biology, or what you will) is represented also by many fine studies on micro-organisms — those of Wyckoff and co-workers on spirochaetes and on the action of bacteriophages, for instance, and others on flagellates, sperm and so on, such as are to be described in papers communicated to this Conference. All these would have pleased Leeuwenhoek mightily, I am sure; but where, as I said a few minutes ago, we electron microscopists most experience the counterpart of his sensations of wonder and discovery is when we penetrate into the underworld of the molecules themselves. Wyckoff, by virtue of the epoch-making shadowing technique he developed with Williams, has been the chief pioneer, and we all remember his first pictures of haemocyanin molecules lying there just like marbles that we could pick up and put in our pockets. Even before that, however, the supreme distinction of being the very first macromolecule to be perceived by the human eye had already gone to the tobacco mosaic virus prepared by Stanley. To me it is not possible to look upon electron micrographs of tobacco mosaic virus without also thinking upon this dramatic moment in the history of science.

As a crystallographer, I am inspired too — we must all be inspired — by the way the electron microscope

portrays an increasing number of viruses in the act of building crystal lattices. The old familiar diagrams of hexagonal close-packing and the like in crystallographic text-books have now become alive — it is what I like to call 'crystallography come true'. Not that X-ray analysis ever left any room for doubt in the underlying reality of these things, but we humans still love the solid satisfaction of seeing things by eye. Crystallographically speaking, though, that is the least of the matter; what is much more important is that by means of this new 'visual crystallography' we are now enabled to shorten, or at any rate confirm, the procedure of X-ray analysis by observing the type, and measuring the dimensions, of certain macromolecular lattices directly. Fig. 2, Wyckoff's micrograph and model of the crystal lattice formed by a necrosis virus protein, is an impressive example of recent achievements along these lines. Another is the turnip yellow mosaic virus studied at Cambridge, which turns out to crystallise in a lattice of the classical diamond type.

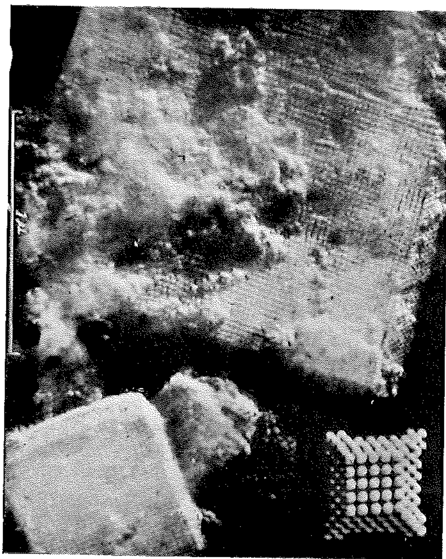


Fig. 2. Cubo-octahedra of a necrosis virus protein, with model of cubic close-packing to illustrate the face development. (Wyckoff).

The mere recognition of such smallest disease-producing particles, many of which appear to be genuine macromolecules, is of course of tremendous value diagnostically long before any possible fulfilment of more fundamental structural aims. The search has gone forward in a great leap since the advent of the electron microscope, and with the help also of ultra-centrifugation and other separative techniques we have now attained a commanding vantage point in the problem of following just what particulate factors and their transformation products are at the bottom of transmissible diseases. In this connection I may be allowed to mention some work we have been engaged on at Leeds in collaboration with the Cancer Research Department there. There is a famous mouse mammary cancer associated with the name of Bittner, who traced it to a virus-like factor transmitted in the mother's milk. Fig. 3 is one of many electron micrographs we have obtained of particles invariably found in extracts of such mammary tumours, and though the parallel biological tests are still incomplete, the evidence to date is very promising that the particles thus disclosed are indeed, or at least include, the so-called Bittner milk factor.

Given more time, I could hold forth at some length on the electron microscopy of viruses, as you know. I could describe, for example, the work of Dawson and McFarlane on vaccinia, and the inter-related findings of Mosley and Wyckoff and of Dawson and Elford on the influenza virus, and many other investigations of the kind; but because the time I may allot to any particular application is strictly rationed, I propose to move on, with similar limitations, to the wide domain that may be called micro-reactions. Here we strive to observe, if possible, changes in the very molecules — only the giant molecules so far, to be sure, but nevertheless some exceedingly significant studies are now in progress. Typical of these, in the field of micro-biochemistry, are current researches into the mechanism of muscular activity, the object of which is to characterise more precisely the proteins myosin and actin (see, for example, Fig. 7) and the complex they form, actomyosin, that is believed to be the contractile agent proper; and to find out what happens to these essential components when they are subjected under various conditions *in vitro* to the action of accessory muscle constituents such as adenosine triphosphate. In common with workers in other laboratories, we have devoted a lot of time to these questions at Leeds, as also to the interpretation of that uncommonly interesting protein tropomyosin, recently discovered in muscle by Bailey. Another outstandingly important biochemical reaction that has been and is still being intensively studied in the electron microscope is the blood-clotting transformation whereby fibrinogen, in the presence of thrombin, passes over into the closely related but strongly fibrous product, fibrin.

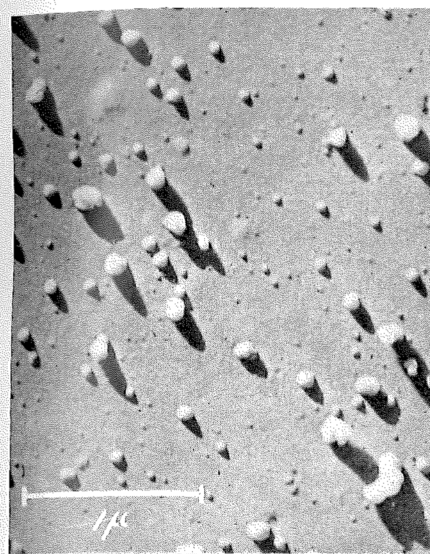


Fig. 3. Particles found in extract of C3H mouse mammary tumor. (Passey, Dmochowski, Astbury and Reed).

Micro-reactions bring me automatically to micro-staining or, as it has been called, 'electron staining'. I suppose the historic application of this technique is the magnificent picture by Hall, Jakus and Schmitt of paramyosin stained with phosphotungstic acid, but there are now

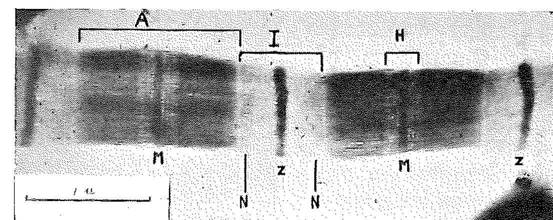


Fig. 4. Toad myofibril stained with phosphomolybdic acid. (Draper and Hodge).

plenty of other excellent results I could cite. One of the most beautiful is the micrograph lately obtained by Draper and Hodge of a toad myofibril stained with phosphomolybdic acid (Fig. 4). This picture not only brings out with exceptional clarity the various muscle bands already recognised, it also reveals for the first time a continuous series of fine striations spaced at about 400 A.U. In view of the present belief that the finest micro-fibrils seen in muscle micrographs are threads of the contractile complex actomyosin, and considering also recent X-ray measurements of the fibre period of actin, it is tempting to infer that these new striations are an expression of the manner in which actin lies along the myofibrils in parallel with myosin.

The region of micro-periods in electron microscopy (corresponding to macro-periods in X-ray diffraction analysis) is obviously another topic on which we might justifiably spend hours of discussion. Take collagen alone, the prototype but still one of the most striking of cross-striated fibrillar structures: we could very easily devote to it a whole course of lectures. In fact, it is probable that the most fundamentally promising field in electron microscopy today is where it overlaps X-ray diffraction analysis. It is a truism in science, of course, that the most fruitful discoveries are to be expected where different disciplines converge, and from our standpoint here we could hardly find a better illustration of this principle than that afforded by coordinated X-ray and electron microscope studies of muscle and collagen. Another development along similar lines that begins to look very hopeful is micro-incineration of the specimen by putting up the intensity of the electron beam. Draper and Hodge have obtained in this way particularly good results with muscle fibrils: incombustible cross-striations remain from the periodic distribution of inorganic constituents, and pretty soon it should be possible to identify the latter by localised electron diffraction photographs.

Before it is too late, I really must say a few words about the master 'application' of all, that of biogenesis. In the last resort, all biological investigations are directed, consciously or unconsciously, to the solution of this problem, and it is something that we electron microscopists, with the new power we have of watching even molecules come into existence, should keep steadfastly in mind. Already certain systematic approaches have been made — witness the work of Wyckoff and his collaborators on the genesis of bacteriophages, and the study by Reed and Rudall of the evolution of the earthworm's cuticle

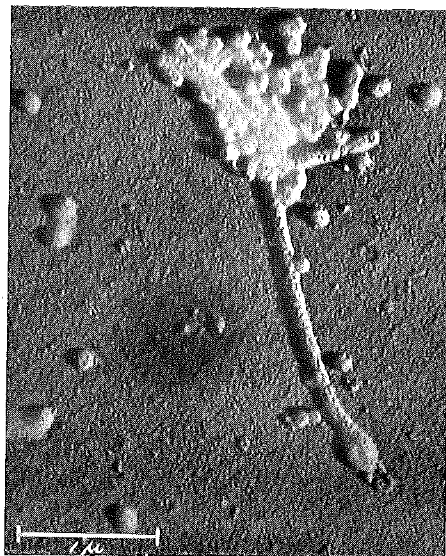


Fig. 5. Forms of influenza virus. (Wyckoff).

— and some further relevant observations, such as on the peculiar filamentous growth-forms of influenza virus and the like, have been reported by Wyckoff (Fig. 5), Bang, Dawson and Elford, and others; but these are only the tantalising beginning; the heart of the matter is still a close secret. Some part of this secret, as it concerns the plant kingdom, is implicit in the very recent surprising revelations on the texture of native cellulose. An electron microscope study by Preston, Nicolai, Reed and Millard showed that the cell wall of the alga, *Valonia ventricosa*, is composed not of cellulose fibrils of all sorts of thicknesses but of a system of remarkably uniform wire-like strands; and Frey-Wyssling, Wyckoff and Mühlethaler have found an essentially similar construction in a number of other plant cell walls, and even in bacterial cellulose (Fig. 6). The cellulose 'wires' have apparently always the same

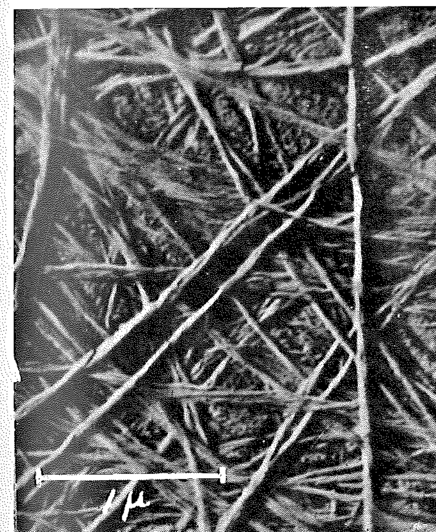


Fig. 6. Bacterial cellulose. (Mühlethaler).

thickness (c. 250 A.U.), suggesting perhaps a common origin in the linear union of initially particulate bodies after the manner of formation of tropomyosin fibrils, of F-actin

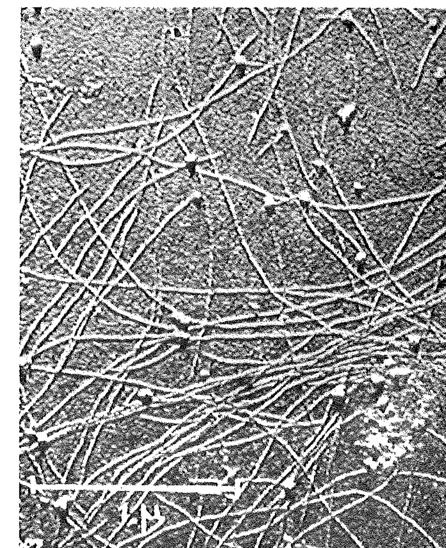


Fig. 7. Micro-fibrils of F-actin. (Jakus and Hall).

(Fig. 7) from G-actin, and of the filamentous growths of Newcastle disease virus (Bang).

I wanted to include in this talk something about non-biological applications; but chiefly for lack of time but partly also because (in the sense that biological preparations are both more varied and on the whole more difficult to deal with technically) the greater includes the less, I shall make the briefest final reference to metals only. It would be inexcusable not to mention metals at least, even though it seems that such advances as have been reported are hardly so spectacular as may have been hoped, and I shall save my face by drawing attention to some recent work by Cuckow. He has constructed, for the

purpose of bringing increased contrast to the usual optical methods of examining the contours of metal surfaces, a phase contrast incident-light microscope and compared its

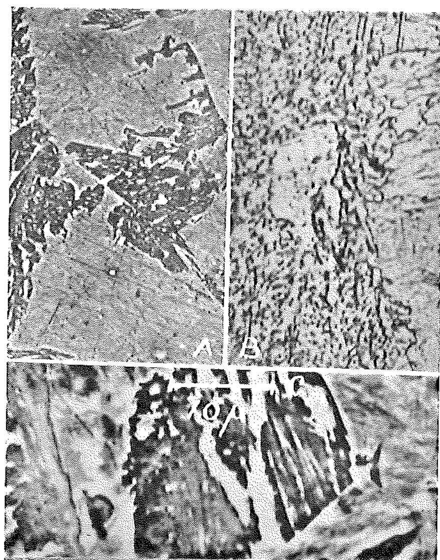


Fig. 8. Ni-Cr steel intermediate transformation products. Comparison between electron micrograph of plastic-film replica (A), normal photomicrograph (B) and phase-contrast photomicrograph (C). (Cuckow).

possibilities with what can be achieved by examining thin replicas in the electron microscope (see Fig. 8). As Cuckow points out, there is an interesting formal analogy between the two methods, and he advocates the time-saving and other advantages of using them in conjunction, the optical procedure for general survey and the electron microscope for higher resolution.

My time has now come to an end, and all that remains is to express the hope which all of us must cherish that Gabor's ideas, or something corresponding, will soon make it possible to find out more and more about *still* less and less.

Discussion.

Ir Le Poole: One of the bacteria pictures showed remarkable contrast of the flagella. How was this contrast obtained?

Answer by Dr Reed: The specimen was shadowcast at grazing incidence. Further experiments showed that this method might be very useful in many cases.

A DIFFRACTION APPARATUS.

M. VAN MENTS and J. B. LE POOLE, T.P.D., E.M.Div., Delft.

The aim of a diffraction apparatus is the study of regular periods in the structure of matter.

If a material is struck by a parallel beam of short wave radiation, *e.g.* fast electrons, the radiation is diffracted. The diffraction pattern (Debye-Scherrer rings or separate spots) can be caught on a fluorescent screen.

In the case of fast electrons the diameter of the rings depends on the accelerating voltage. If, therefore, this voltage is not stabilized the rings have a variable size which might blurr the image and spoil the accuracy.

Instead of using a complicated and expensive stabilizer an elegant method to get rid of these chromatic errors may be applied. For this purpose the focussing lens is put between object and screen instead of in its normal place between gun and object. Calculation shows that the position and the strength of this weak magnetic lens must be chosen so that the ring diameter is reduced by one third.

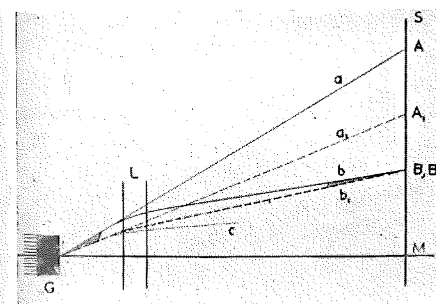


Fig. 1.

The effect of this lens follows from Fig. 1. For better demonstration the angles and dimensions are exaggerated. Let "a" be a diffracted beam at normal voltage striking the screen S at A.

If the voltage is increased the beam is diffracted at a smaller angle. The trajectory has become *e.g.* "a_1" impinging on the screen at A_1, AA_1 is called the chromatic error.

With a weak magnetic lens between object and screen the beam is bent towards the axis. Trajectory "a" is deflected to "b", hitting the screen at B.

If the magnetic lens would have the same power at the higher voltage „a_1" would get the direction "c".

But the lens shows chromatic aberration, *i.e.* its power is less at higher tension, so that "a_1" becomes "b_1" arriving at the screen in B_1.

It follows from simple calculations that $BB_1 = 0$ if $BM = 2/3$ AM in this simple case.

Unfortunately the magnetic lens introduces a rotation which depends on the voltage. In Debye-Scherrer

diagrams the rotation is of no interest, but this is different in the case of single crystal patterns. It is necessary therefore to prevent rotation by using at least two lenses with their fields in opposite directions as indicated in Fig. 1, by the two lines at L.

Now there is one error left which needs careful consideration, *i.e.* distortion. Distortion is caused by the fact that marginal rays are bent too much. This means that the reduction in size of the pattern is not the same over the whole of the picture. The outer rings become too small.

Since two lenses are needed anyhow to prevent rotation, the second one might as well be used to correct the distortion of the first. This can be done by using two slightly stronger lenses at a bigger distance from each other and having the electron trajectories cross the axis before reaching the screen (Fig. 2).

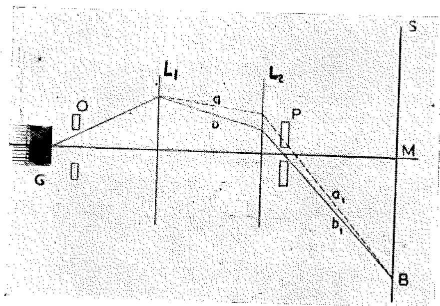


Fig. 2.

It is clear that in this new situation the distortion of the second lens is counteracting that of the first as an increase in power of this second lens results in an increase in size of the pattern.

If the chromatic error and the distortion of both lenses are known it is possible to correct these errors in the pattern by careful choice of position, dimensions and power of the lenses.

In Fig. 2 the "paraxial" ray is given by aa_1 and the actual ray by bb_1. The large amount of freedom in the position of the lenses made it possible to have the ringdiameter also independent of the position of the object. This is important in the case of reflection patterns where it is difficult to determine the exact spot where the beam strikes the specimen.

Once this arrangement is given, it is possible to put a lens (projector) with a small bore at the crossover, and another lens (objective) close to the specimen. With these two lenses energised the apparatus is used as a microscope.

Along the lines given, a combined electron diffractograph-microscope was designed by the authors at the request of the Royal Dutch Shell. The final set-up and technical arrangements were designed by H. C. Corbet of this company.

The apparatus is now working succesfully. It shows one unanticipated and very convenient feature: By the small aperture of the projector lens the background is apparently screened off which makes the obtained pictures much clearer. The distortion is less than ¼ % (not measurable).

When used as a microscope the magnification is 3000 diameters. If the diffraction lenses are switched on at the same time the magnification is reduced to about 500 diameters.

The reflection patterns are sharper than those obtained with the usual diffractographs.

Discussion.

Mr Haine: What is the angular resolution? If only $^1/_{2500}$ it would appear that

simple high voltage stabilizing would be simpler.

Answer: Accurate measurement of H.T. is difficult owing to changes of the order of 1 % in high voltage resisters over a long period, *e.g.* a couple of months.

Mr Halma: Can the area selected for diffraction be related to the microscopical image?

Answer: The illuminated area shows up in the microscope. Good alignment is necessary.

Dr Cosslett: How difficult is the instrument to operate as there are four lenses to adjust?

Answer: The four lenses are aligned by careful machining. The tilt of the gun is variable. We found no difficulty in operation.

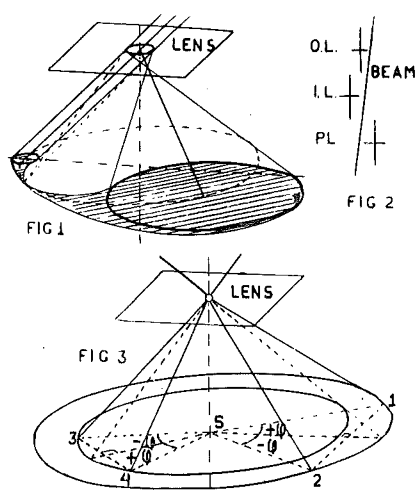
HIGH-TENSION MICROSCOPE ALIGNEMENT.

J. VAN HENGEL and J. KRAMER, T.P.D., E.M.Div., Delft.

The aim of the alignement of a microscope is to adjust the various lenses until all the axes of symmetry coincide.

In order to understand the alignement procedure, we must know what happens to a ray passing through a magnetic lens (Fig. 1). The inclination to the axis of the ray passing through the center — or better through the nodal points — of the lens is not altered by the lens. However, a change in the current will cause the plane through the axis and the ray to rotate.

So this ray through the center will move along a cone and the center



of convergence describes a circle, the center of which lies on the axis.

The alignement starts from the position as given in Fig. 2.

A two-stage microscope is generally aligned by adjusting the inclination of the gun until the center of rotation at a small variation of the objective current and the center of the screen coincide. The effect of a small distance between the axes of objective and projective lenses is compensated by a residual inclination. In our three-stage microscope (with an intermediate lens added to obtain a wide range of magnification) this method *seemed* to be good enough at a given high tension.

Over a wide range, however, *e.g.* from 100 till 300 kV., at every turn a correction of the alignement proved necessary. Therefore we provided the intermediate lens with three adjustment screws.

The projective lens current is set at its normal value, so one looks at what happens in the intermediate image plane at a magnification of about 100 times.

In the method used alignement is obtained in two steps:

a. The beam is directed parallel to the axes of the lenses.

b. The axes are made to coincide.

a. 1. With the objective lens current at zero an image is formed of

the electron source with the intermediate lens. This image is formed on the screen *e.g.* in point 1.

2. Reverse the current in the intermediate lens: image now in point 2. The axis must lie in the symmetry plane of 1 and 2.

3. Change the inclination of the beam. Suppose the image is now in point 3.

4. Reverse current again: image in point 4. Again the axis must lie in the symmetry plane of 3 and 4, and is therefore the intersection of these two symmetry planes (see point 5).

The significance of the several points is illustrated by Fig. 3.

b. Move point 5 to the center of the field of view by means of the intermediate lens adjustment screws in order to obtain the desired coincidence of the axes of the projective and intermediate lenses.

Finally the objective lens should be adjusted. Set the objective lens current at a rather high value. By this one gets a cross over very close to the lens and to the axis. With the intermediate lens an image of this cross over is made. Move this image to the center of the field of view by means of the objective adjustment screws. The alignement of the microscope *sensu stricto* (without accelerating tube and condensor) is now completed.

THE PHILIPS ELECTRON MICROSCOPE, TYPE 11980.

A. C. VAN DORSTEN, Philips, Eindhoven.

The Philips Electron Microscope as it is in production now, is provided with some devices, which have been tested in practice over a period of two years. Two of these devices will be discussed briefly. They are related with focussing and the obtaining of transmission diffraction patterns. Both methods have been described by J. B. Le Poole.

The *focussing device* employs a magnetic beam deflecting system, mounted in the region between condensor and objective lens. Two sets of coils can be energized by an alternating current of suitable strength; in the first set a periodic deviation of the beam is caused, which is followed by a reverse effect in a second set of coils between the first set and the objective lens. The electrons, after being deflected from the axis and being directed back thereafter, will intersect the optical axis near the object side focal plane of the objective lens under a periodically varying

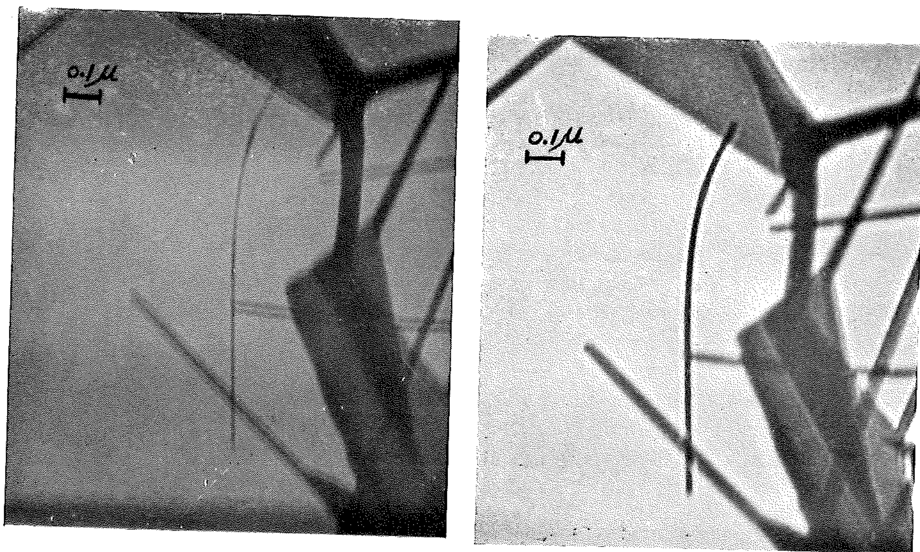


Fig. 1 and. 2. The vertical, slightly bent, crystal needle of ZnO has been focused 1 using the beam deflector, 2 visually. The actual size on the screen during focussing was about 4 times smaller than the size on this picture.

angle, the maximum amplitude of which is about 20 to 30 times the usual aperture angle of illumination. Thus in one plane this angle is enlarged periodically; the beam intensity in the object, however, remains constant.

By a press button operation the coils can be energized at any time during focussing and an out-of-focus image is detected at once, the image being blurred by a more or less pronounced "astigmatism". The method is very sensitive and a great help in focussing images in difficult cases. It is well known that the focussing for maximum contrast is not the focussing for the paraxial image, the latter, however, will give a more true picture of the object. When, in some specimens, details are only visible if use is made of the extra contrast of an out-of-focus image, the "wobbling beam" focussing device is very useful for defining the range of objective lens current settings to be employed for a series of through focus pictures.

Figs. 1 and 2 illustrate the effects described in the foregoing for an object of crystalline nature.

For obtaining of electron diffraction transmission patterns of a selected area out of the specimen *in situ,* the first intermediate image is focussed near the focal plane of the weak intermediate lens, situated between objective and projector lens. An adjustable square aperture limits the area of this intermediate image. The magnification in the first stage is about 30 times and thus the actual size of the aperture corresponds to a 30 times smaller area in the specimen. The excitation of the weak lens is adjusted so as to focus the image side focal plane of the objective lens via an intermediate image in the projector stage on the fluorescent screen. The diffraction image existing in the focal plane of the objective is thus projected on the screen, as far as it corresponds to the area defined by the selector aperture.

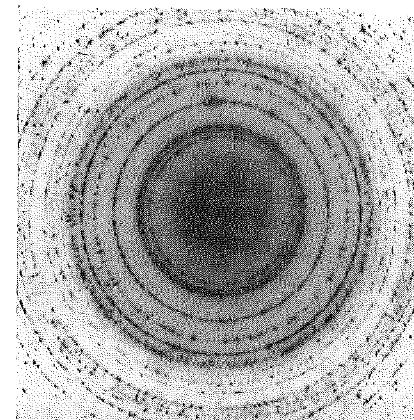


Fig. 3. Electron diffraction pattern of a group of ZnO crystals. Note the radially and otherwise elongated spots. Size on the screen 1½ times the size of this picture.

The normal image is focussed near the image side focal plane of the projector lens and is thus eliminated from the screen. The diffraction patterns obtained in this way can be very rich in details due to the fact that the area concerned in the specimen can be restricted to under 1 micron square and that the depth of field equally is of about the same size. Fig. 3 shows a pattern obtained from a group of ZnO crystals.

References.

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SOME THEORETICAL AND PRACTICAL CONSIDERATIONS OF IMAGE CONTRAST IN THE ELECTRON MICROSCOPE.

J. HILLIER and E. G. RAMBERG, R.C.A. Laboratories Division, Princeton, N. J.

Obviously, without contrast there can be no image, and without adequate contrast there can be no recognition of image detail. Yet we do not often define what adequate contrast is nor examine the factors determining contrast.

A relatively detailed treatment of minimal contrast requirements in the electron microscope image has recently been published by von Borries [1]. In this, and earlier, publications von Borries is particularly concerned with the performance of various photographic materials. A rather general approach to the problem of contrast recognition on *e.g.* a photographic negative may prove helpful in creating a feeling for the practical significance of contrast.

Consider an electron micrograph negative with a patch of diameter D, corresponding to a small object in the specimen field, on a background of uniform density. Let the density of silver grains in the patch be N, that in the background N_0. It is obviously necessary that the difference in the number of grains in the patch and the average number of grains in an equal area of the background should considerably exceed the average statistical fluctuation in this number; measurements by Schade [2, 3] indicate that the ratio of the difference to the mean deviation should be of the order of 5. We thus find

$$\frac{D^2\,(N_0-N)}{\sqrt{D^2\,N_0}} = \frac{Dk\,(I_0-I)}{\sqrt{I_0-N_f/k}} \geqq 5$$

Here I_0 and I are the charge densities incident on the background and the patch, respectively, N_f is the density of fog grain, and k is the number of grains rendered developable per unit charge incident on the plate. This formula applies as long as there is no appreciable overlap of grains. It shows that

1. The effective plate sensitivity (for fixed fog grain concentration) is determined only by the average number of grains rendered developable per incident electron.
2. The recognition of contrast improves with exposure until there is appreciable overlap of grains.
3. The minimum intensity difference between object and background required for detection is inversely proportional to the diameter of the object.

The first statement leads to a contrast figure of merit of a photographic emulsion which is identical

with that for resolution *if* — and this is a big if — resolution is limited by grain size and not by electron diffusion in the emulsion. Diffusion does not play as important a role in contrast recognition as for resolution, so that relatively thick fine-grained emulsions may be advantageous for particle detection even if their resolution, as evinced by the diffusion of sharp edges between light and dark areas, is poor.

Consider now the production of contrast in the electron image by the action of the specimen and the imaging system on the incident electron beam. If a perfect imaging system with a given physical aperture could be assumed, the intensity variation in the image would be determined uniquely by the scattering characteristics of the object; true electron absorption is relatively insignificant. The effective transmission coefficient of an element of the specimen is simply related to the fractional scattering cross section of elements of area of the specimen: the fractional scattering cross section is the ratio of scattering processes through angles greater than the aperture angle to the total number of incident electrons.

Atomic scattering cross section formulas have been published, some time ago, by Marton and Schiff [4]. Here the elastic cross sections for light atoms are calculated with Hartree functions, for heavy atoms, with the Thomas-Fermi model, and inelastic cross sections are determined with Slater wave functions. In addition, the contributions of the free electrons in metals are determined with the aid of the free electron scattering formula. These formulas have been employed to obtain the curves for the fractional cross sections of films of identical mass density, composed of different elements and bombarded by electrons of different energies, which are

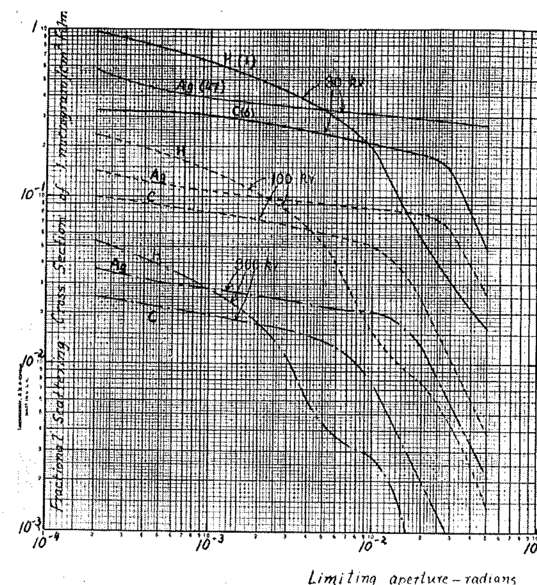

Fig. 1. Fractional scattering cross sections of films composed of different elements as function of angular aperture of objective.

shown in Fig. 1. The employment of more accurate formulas for atomic scattering cross sections, such as have recently been published by von Borries [5], would, in particular, lead to a more gradual transition from the region of slow variation of scattering cross section with aperture angle to that of very rapid variation at large angles.

Comparing the cross sections for equal mass densities of different elements at a fixed voltage (*e.g.* 100 kV) we find the following picture: For all except the lightest elements the scattering cross section varies little over the practically important range, from several ten thousandths of a radian to over a hundredth of a radian. For larger angles the scattering cross section falls off sharply, as the inverse square of the aperture angle, the knee of the curve moving toward larger angles with the 4/3 power of the atomic number. This behaviour is dominated by the elastic scattering at the nucleus of the atom. For lighter atoms, and hydrogen in particular, the inelastic scattering at the atomic electrons predominates and causes a gradual increase in the scattering cross section with decreasing aperture angle right up to angles of the order of 10^{-4}. Finally, there is an upturn of the scattering cross section curve for metals at very small angles occasioned by the conduction electrons.

We see that reducing the aperture below the effective aperture for optimum resolving power, that is, below 10^{-2} radian, has only slight effect on the contrast produced by the heavier elements. In fact, if we should build heavy atoms into organic compounds so as to bring them out more clearly, we would find reduced contrast with decreased aperture. A very small aperture favors hydrogen and, in particular, very light metals such as beryllium.

If the operating voltage of the electron microscope is increased, the scattering cross sections decrease and the knee of the scattering curves shifts to smaller angles. Since, however, the optimum operating aperture also shifts to smaller angles, the scattering cross sections of the heavier elements remain relatively insensitive to aperture angle in the range of greatest interest.

The curves shown in Fig. 1 apply for disordered atoms; if the atoms are arranged in lattices, there will be strong scattering for certain orientations of the lattice relative to the incident beam. Boersch [6] has shown that the ordering produces a strong enhancement of scattering also for thin crystalline layers perpendicular to the beam.

The scattering cross section curves can be employed directly for the prediction of contrast only in systems with a physical objective aperture of the same order as or smaller than the optimum objective aperture. This mode of operation has the practical drawback, that the fine aperture required is readily contaminated, leading to asymmetries and charging shifts in the image.

With the wide open objective, the curves will still give a roughly correct idea of detail contrast if the concentration of scattering material in the region imaged is minimized and/or the illuminated area of the specimen is made small. Suitable objects are isolated fine particles or thin fibers. With parallel illumination, maximum contrast will be observed at the specimen edges. This edge contrast enhancement follows equally from the presence of aberrations and from defocusing. A more detailed examination reveals the presence of diffraction fringes, whose asymmetrical character with respect to the direction of defocusing is explained by refraction of the electron beam by the specimen [7]. Kinder and Recknagel [8] have demonstrated this phenomenon by model experiments with light. The index of refraction is given by the inner potential of the film and may, for 50-kV electrons and a film thickness of 100 A.U., lead to a phase delay of the order of $\pi/2$.

With parallel illumination the edge contrast produced by the phase delay in the film may well outbalance that determined from the scattering cross section curves [9]. More important, for too high lens currents objects appear surrounded by a dark fringe whose disappearance marks the position of optimum focus. Optimum focus — from the point of view of resolution — unfortunately is also characterized by minimum edge contrast.

We have considered quite generally the special case that the specimen is sufficiently thin that the contrast may be determined by single-scattering considerations; it may be shown that this applies for organic material of unity mass density provided that the thickness is less than 0.3 micron at 50-kV. It is doubtful whether the electron microscope can be employed to great advantage for much thicker materials, even if, by the employment of high voltages, adequate transparency is obtained: If the thickness is a large multiple of the structure which is to be resolved, volume scattering and the superposition of intensity distributions from different levels of the object combine to render the interpretation of the observed pattern difficult and inconclusive.

An experimental check of the conclusions regarding contrast which have been reached is rendered difficult by several factors. One of these is the large influence of photographic processing, which is rarely adequately standardized. A second is the indirect effect of lens astigmatism: Even today the great majority of electron microscope objectives in use have appreciable astigmatism; this is generally accentuated when a limiting aperture is employed. The microscopist with an astigmatic objective will be forced to underfocus in order to obtain an acceptable image, with consequent accentuation of edge contrast.

A clean, perfectly symmetrical aperture will have little effect on the detail contrast and resolution in the image formed by a stigmatic objective, provided that its diameter is of the same order as or greater than that of the optimum aperture. At perfect focus images quite generally appear flat, even for specimens such as colloidal gold (Fig. 2). In specimens

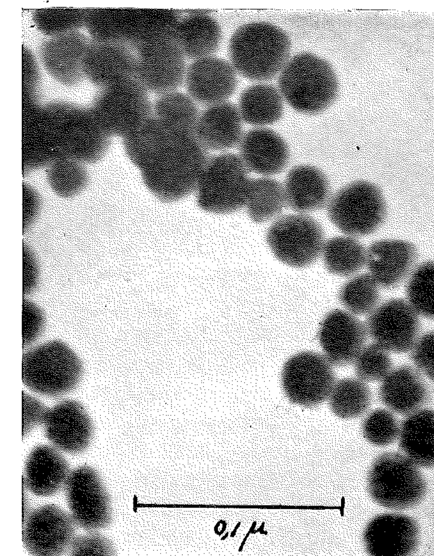

Fig. 2. Colloidal gold. In-focus micrograph taken with symmetrical objective.

which have considerable extension in depth, such as carbon black, the character of the fringes may vary from particle to particle, but is throughout symmetrical. The visibility of internal structure in the grains can be taken as a sign of good instrument adjustment (Fig. 3).

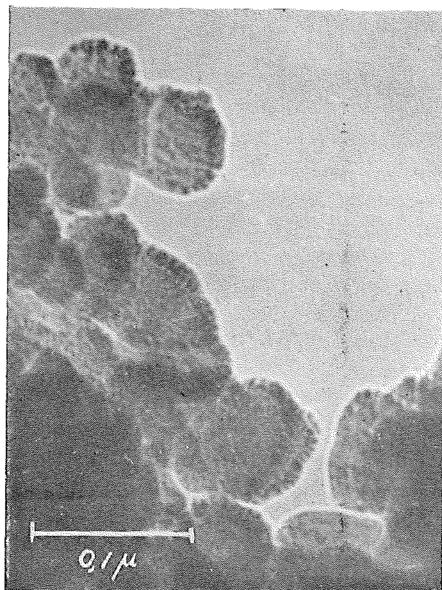

Fig. 3. Carbon black, showing internal structure.

Contrast can be enhanced very considerably, particularly in organic specimens, by the employment of very small limiting apertures; the preparation and insertion of the necessary apertures is facilitated by the employment of a double objective with long-focus front lens [10]. Thus Fig. 4 shows identical portions of a shadowed tissue cell imaged with and without an aperture limiting the accepted cone to 10^{-3} radian. In spite of the enormous difference in contrast in the two pictures, there is no great difference in the information supplied: a careful examination indicates that the thinner portions of the specimen show more detail in the image taken without the aperture, the thicker portions, more in that taken with the aperture. With thick specimens the gain in contrast resulting from the use of apertures even of relatively large dimensions is often striking. This is, however, generally mainly gross contrast and is not accompanied by a material improvement in the resolution of detail. In any case, for thin specimens, the increase in contrast and the gain in information obtainable by the employment of physical apertures rarely outweighs the technical difficulties attendant on introducing a clean, perfectly centered objective aperture into the microscope. For very thick specimens, over 0.2 micron, the advantage gained is reduced by the limited resolution that can here be obtained.

A more promising approach to the problem of increasing contrast consists in appropriate modification of the specimen preparation technique. This is the course taken by Knaysi [11] in studying the life cycle of *B. mycoides*. By germinating the bacteria on a nitrogen-free nutrient, the food storage in the body of the cells was so depleted, that structural details in the interior were clearly outlined.

Staining with heavy-metal salts is another valuable method of enhancing contrast. Phosphomolybdic and phosphotungstic acid have been found particularly effective, although their deposition on organic material seems to be generally nonselective.

Shadow casting as a method of contrast enhancement was first suggested and demonstrated by H. O. Müller [12] and H. Mahl [13] and has since been developed into an exceedingly useful technique by Williams and Wyckoff [14]. Metal atoms evaporated onto the specimen obliquely produce a deposit of high scattering power whose distribution corresponds rather precisely to the intensity of illumination produced by a source of light placed in the position of the source of the metal atoms. The method is inherently inapplicable to revealing internal structure in organic material.

Sectioning, as developed by Pease and Baker [15] at the University of Southern California, makes possible the selection of undisturbed structural detail from a mass which is too large for effective direct observation with the electron microscope. It enhances contrast by removing irrelevant superposed structures.

The employment of etching is as yet largely unexplored. While the employment of enzymes for this purpose generally weakens the organic structures so that they cannot withstand the subsequent drying process, the use of ion bombardment within the electron microscope holds considerable promise.

Replication, eventually combined with shadow casting, is a final effective method of securing details of external structure of any material which is too thick to be satisfactorily imaged by transmission.

Summarizing, we may state that the attainment of sufficient contrast provides a major practical limitation in electron microscopy today, particularly in the observation of organic specimens. Here the resolving power of the instrument far exceeds the resolution in fact achieved. Purely electron-optical methods of enhancing contrast, such as the

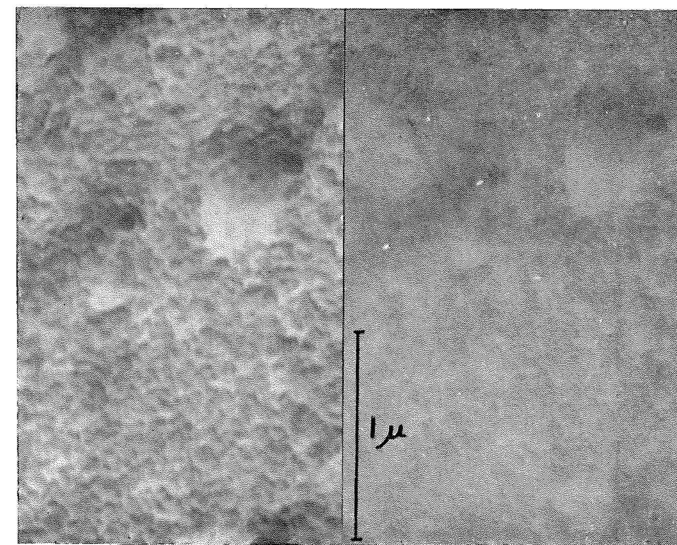

Fig. 4. Detail of shadowed cultured tissue cell.

a. Soft print of micrograph taken with objective of 10^{-3} radians.

b. Hard print of micrograph taken without objective aperture.

employment of small apertures, show little promise of bridging the gap. Hence we must rest our hopes primarily on the development of suitable techniques of specimen treatment. Specimen preparation, staining, shadow casting, sectioning, and etching, in particular, present practical avenues of contrast enhancement. All of them require much further work before their full possibilities can be realized.

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Discussion.

Dr Liebmann: Several curves of the calculated contrast ratio as function of aperture were shown, based on a formula by Marton and Schiff. Is any experimental work known confirming these formulae, or has one to rely on calculation only?

Answer: Experimental data on scattering at the very small angles which are here of greatest interest are virtually nonexistent.

Dr Cosslett: Is there not an intermediate condition of working, between using a fine aperture (and losing resolution, whilst gaining contrast), and using no aperture (gaining resolution, and losing contrast)? This agrees with Ramberg's scattering factor curves, where there is a great drop in contrast between 10^{-1} and 10^{-2} radian.

Answer: A relatively large aperture will give improved gross contrast, but little improvement in detail contrast or gain in information, particularly with a thin specimen. If the aperture introduces astigmatism, the resolution will be unfavorably affected.

SHADOW CASTING.

A. J. A. NIEUWENHUIJS, T.P.D., E.M.Div., Delft.

The differences in scattering power in the specimen are often insufficient to use the resolution of the instrument to its full extent. The shadow casting technique, first introduced by H. O. Müller in 1941 and worked out by Williams and Wyckoff in 1944, has become an indispensible help to increase the contrast. Unshadowed polystyrene spheres with a diameter of 2500 A.U. can easily be seen, but particles of organic material smaller than about 200 A.U. are only visible when shadowed.

By shadow casting we gain more than the increase in contrast alone, as the three dimensional effect of the micrographs facilitates the interpretation.

In this study attention has been paid to various shadowing materials and to the vacuum needed for the evaporation of the metal. Evaporation took place at 16 cm. from the specimen and shadows were cast at 1 : 4 angle.

A vacuum of 5.10^{-4} mm. Hg does not suffice to procure sharp contours to the shadow. On the background small spheres are seen with a diameter from 100 A.U. down to 20 A.U. Some have a shadow and some have not. This background pattern is due to the structure of the collodion revealed by the shadow casting and to the agglomeration of the gold layer which is caused by the impact of the beam from the biased gun. Agglomeration starts at a critical intensity of the electron beam, namely at a current density of about 2,5 times 10^{-4} Amp./cm^2. Brightness at 20.000 diameters is then just sufficient for focussing. Small impurities on the collodion membrane may stimulate the agglomeration. Especially a gold shadow on clay particles shows this effect. The tendency of gold to agglomerate and the collodion structure make it hardly possible to get better than 100 A.U. useful resolution.

The problem is to prevent this agglomeration and to find a background with no structure of its own. There is no difference worth mentioning between the collodion membranes made according to the well known techniques, and formvar membranes.

To keep the shadow free from metal, which might give rise to a wrong interpretation when we are dealing with particles smaller than about 100 A.U., the vacuum in the shadow casting apparatus should be 10^{-5} mm. Even then some structurated details are observed inside the area which lies in the shadow

of the spheres. In the same area nothing was visible before the metal had been evaporated.

The gold agglomeration does not take place simultaneously on the collodion film and on the polystyrene spheres. When the beam intensity increases the gold agglomerates on the polystyrene as well.

To prevent structures in the background, a preshadowed replica technique has been worket out by Williams and Wyckoff. The gold agglomerates but no membrane structure is seen as only the glasss surface was shadow cast. Attempts have been made, though without much success, to reduce this agglomeration in preshadowed replicas. Recently Williams reported results by using Pt-Pd alloy as a preshadowing material. Details down to 20 A.U. could be made visible. The high temperature needed for the evaporation, and the formation of an alloy with the filament make this technique difficult for routine work.

Some metals do not agglomerate by the impact of the electron beam, but unfortunately these cannot be stripped from glass. There seems to be a correlation between the tendency of metal films to agglomerate on plastic membranes and the possibility to strip these films from glass. Such as oxidise readily do not show agglomeration but cannot be stripped from a glass surface, which is the smoothest surface available. Chromium only slightly tends to agglomerate. Owing to the low scattering power of this element a layer of 60 A.U. is needed for sufficient contrast. So in the direction from which the metal was evaporated the thickness is 240 A.U. To determine the diameter of the particles one should measure in a direction perpendicular to the direction of the shadow. To bring out contours of relatively large objects such as bacteria chromium can be used as a shadowing metal. Small details on the surface down to 100 A.U. can be seen though one should remember that distortion takes place in consequence of the heavy layer of chromium.

Also useful, though more difficult to evaporate, are Pd and U. Pd alloys with the tungsten wire and has a slight tendency to agglomerate. For sufficient contrast the layer should be 20 A.U. thick. With U we need only 4 A.U.

Far more convenient is the use of a mixture of gold and manganine resistance wire, as reported by Le Poole. These metals can easily be evaporated simultaneously. A thickness of 12 A.U. is sufficient. Agglomeration shows up only at very high beam intensity. Maybe Mn, by its tendency to oxidise, forms bonds with the oxygen atoms in the membrane thus anchoring the metal layer. This would agree with the fact that it cannot be stripped from glass.

In order to use the instrument to its full extent it is still necessary to pay attention to these problems.

Discussion.

Mr Agar: We have found that a 40 % gold-palladium alloy does not aggregate under a beam from a biased electron gun. This metal allows of preshadowed replicas being easily stripped from glass.

Dr Cosslett: Aggregation does occur on latex sometimes even to a single blot on one side. This observation concerns gold-palladium, so even this mixture can be aggregated

Dr Brown: Mr Nieuwenhuijs said "the oxidisable metals are more difficult to strip". Evaporated aluminium can be stripped readily from anything, not only glass, but even metals.

Answer: Up till now we could not strip the oxidisable metals from glass. Therefore I suggested that these metals might form bonds with the oxygen from the glass. That

aluminium can be stripped from metals is not in contradiction with this suggestion. That thin Al films can be stripped from glass is new to me. Unfortunately the scattering power is insufficient for use as preshadowing material.

Dr McFarlane: Why is there so little aggregation of the metal on the surface of the polystyrene particles? In principle one would expect that to be most marked here; may it be present but invisible because of the low electron transmission of the particles themselves?

Answer: The exact mechanism of the aggregation is not yet clear. The surface on which the metal is evaporated as well as the energy absorbing properties of the object will effect the aggregation. From experiments we know that these are two independent factors. The micrographs show that aggregation on polystyrene spheres starts later than the aggregation in the background. The particles are transparent in the micrographs as we are using an acceleration voltage of 100 kV.

Dr Reis: Les particules métalliques arrivent sur la préparation à ombrer sous des angles légèrement différents. Quelques essais préliminaires que M. Robillard et moi-même avons faits dans mon laboratoire ont montré que la netteté des bords des ombres est augmentée si on se sert de diaphragmes donnant un rayonnement ombrant parallèle. Je voudrais savoir si de tels essais ont été faits par M. Nieuwenhuijs?

Answer: We have not tried the aperture method for getting sharper shadows. If the vacuum is good enough we get sufficiently sharp contours, so it does not seem worthwhile to go through all the trouble of aligning.

A PRELIMINARY REPORT ON ATTEMPTS TO REALISE A PHASE CONTRAST ELECTRON MICROSCOPE.

A. W. AGAR, R. S. M. REVELL, Metropolitan Vickers Electrical Company, Research Dept., Manchester and R. A. SCOTT, Henry Simon Ltd.

This paper describes an attempt to use the electron microscope under phase contrast conditions. The system used has been suggested independently by Boersch and Gabor, and is analogous to that introduced by Zernike in 1934 for the optical microscope.

In the case of the electron microscope the phase plate takes the form of a suitably perforated thin film. A lower limit is placed on the size of the hole in the film by the spreading of the focal spot behind the objective, associated with the inevitable slight divergence of the illuminating beam in the conventional electron microscope. The size of this focal spot in the Metrovick Type E.M. 2 microscope used in these experiments is about 0.6 μ and this is therefore the optimum size for the phase plate hole. Owing to the experimental difficulties, however, the size adopted was 1 μ. Such a hole transmits unchanged the central portion of the diffraction pattern and consequently the phase delay will be applied only to those parts of the pattern containing information on the fine detail in the object. For a 1 micron hole, 50 kV. electrons and a 4 mm. objective, the upper limit of size for which phase contrast can be expected is 400 A.U.

Requirements of the Film.

The film thickness must be such as to introduce a phase delay of $\pi/_2$ in the illumination passing through it. Thus if μ is its effective refractive index, and λ the wavelength of the illumination

$$(\mu-1)\ d = \frac{\lambda}{4}; \qquad \mu = \sqrt{\frac{V+V_1}{V}}$$

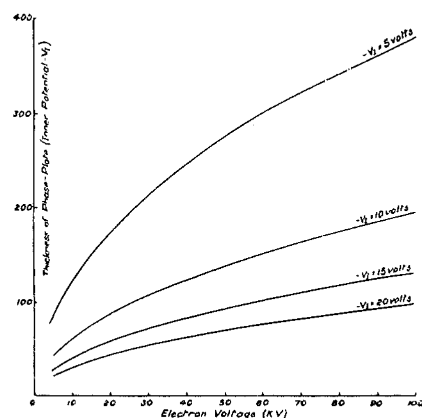

Fig. 1.

where V is the accelerating voltage and V_1 is the inner potential of the material of the film. No direct measure of V_1 exists for amorphous materials such as were used, but it can be reasonably assumed to lie within the range determined for crystalline materials, *viz.*, 1—20 volts. Typical film thicknesses for different values of V and V_1 are plotted in the curves of Fig. 1. Films of the requisite thickness are readily prepared and their thickness can be estimated to an accuracy of about 5 % by a multiple beam interference method.

After experimenting with a number of materials, formvar was chosen for these preliminary experiments, as being fairly strong and of low electron scattering power and uniform thickness.

Formation of Aperture.

It was found that naturally occurring holes in film usually showed thickening around the periphery, and perforations made from a spark coil were rather uncontrollable. A mechanical piercing method was therefore adopted.

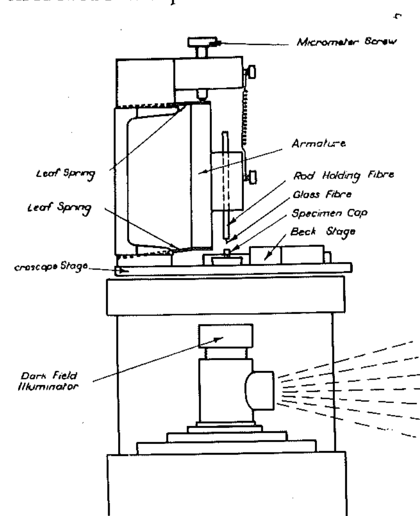

Fig. 2.

A glass fibre with its point etched to rather less than 1 μ diameter was mounted on the stage of a projection microscope in the apparatus shown in Fig. 2. This enables the film material to be viewed as the fibre point is depressed by the micrometer screw, and holes down to 1 micron can be made in the film with a considerable accuracy in positioning.

Positioning of Aperture.

When the microscope is set up for phase contrast working, the hole in the phase plate must be centred on the crossover with an accuracy of a fraction of a micron. This was achieved by constructing a phase plate stage, shown diagrammatically in Fig. 3. This enabled the phase plate to be traversed in any direction in its plane by two controls outside the microscope.

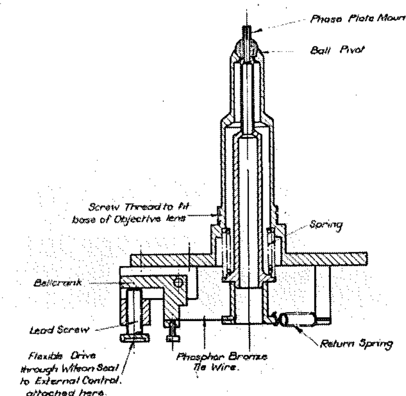

Fig. 3.

In order to facilitate the adjustment, arrangements were made to view the phase plate with an auxiliary lens fitted in the microscope immediately below the phase plate mechanism. This enabled the phase plate hole of 1 micron diameter to be seen as an image 0.5 mm. across on the final screen.

Adjustment of Illumination System.

In order to maintain phase contrast conditions in the coarsest possible structural detail, it was necessary to take extreme measures to minimise the size of the image of the source. A calculation shows that the condenser must be strongly overfocussed if it is to be possible to obtain phase contrast even in elements of detail as coarse as 400 A.U. Consequently the illumination intensity is much lower than in normal working. A further limitation on beam intensity is set by the strength of the phase plate film, which has to withstand the intensity of the focussed image of the cross-over. In order to reduce this intensity to workable limits, a disc with a 60μ aperture is used as the object support. With this limitation, and using minimum intensity from the gun, it is possible to avoid destruction of the phase plate. Once it has been accurately positioned, some increase in intensity is permissible.

Test Specimens.

Specimens containing fine structure of low contrast are required as test objects. It was considered that formvar replicas of metal surfaces very lightly etched would be suitable, but large protein molecules or colloidal particles of light elements might also be used.

Experimental Results.

Preliminary experiments have been performed using the techniques described but it is still too early to draw positive conclusions as to the value of this technique, owing to the various experimental difficulties encountered, namely:

a. Owing to the very low intensities which must be used, the focussing of the image becomes very difficult. In addition, the photographic exposures required are between a half and one minute, with consequent danger of disturbance from electrical and thermal instabilities.

b. With the apparatus now in use, the phase contrast effect can only be expected in detail finer than about 400 A.U. which under the conditions of *a* above is not easy to detect.

c. It has been found that a dark deposit quickly forms around the phase plate hole, rendering it in all probability useless for this work. Speed in alignment and photography is therefore essential.

d. It is likely that the resolution of the instrument may be adversely affected by the insertion of the phase plate, in which event the 400 — 30 A.U. possible range of the phase contrast effect may be reduced.

The experiments are being continued with a more efficient electron gun, and it is hoped that the increased intensity available with smaller cross-over size will enable the phase contrast effect to be observed in coarser detail than was possible hitherto.

Discussion.

Ir Le Poole: Is there any objection to using evaporated films? It is easy to make small holes by perpendicular shadow-casting of glass spheres.

Answer: In the early stages of this work we performed experiments using the system suggested by Mr Le Poole. There is no difficulty in principle; the difficulty lies in selecting a suitable material. We used beryllium and silica; both proved to have an excessive scattering power when thick enough to act as quarter wave retarders. In addition beryllium was found to exhibit crystallinity and it was difficult to prepare sufficiently clean silica films.

PROBLEMS AND PROSPECTS OF ELECTRON DIFFRACTION MICROSCOPY.

D. GABOR, Imperial College of Science and Technology, London.

What one might call "orthodox electron microscopy" has practically reached the limit of its development three years ago, when Hillier and Ramberg compensated the astigmatism of their objective, and to all intents and purposes realized the theoretical resolving power. At the same time they demonstrated that there was one more obstacle in the way of further improvements than had been suspected. Even for a resolving power of about 10 A.U. the objective must be round to within about one part in 10,000. If one wished to realize a ten times better resolving power, this would have to be improved to one part in a million, clearly a practical impossibility. In 1947 I hit upon the idea of diffraction microscopy, which was first made public at the London Electron Microscope Conference, in April 1948, with the first experimental results obtained with an optical model. The model experiments have come to a close half a year ago, with fully satisfactory success. The combined electronic-optical scheme has reached an advanced stage of preparation in the Research Laboratories of the Associated Electrical Industries, Aldermaston, but no experiments have yet been carried out. This is perhaps the right moment to review the optical experiments, and to give a preview of the problems and prospects of the electronic work, which is likely to extend over several years.

In diffraction microscopy the image of the microscopic object is obtained in two steps. The first is carried out with electrons, and produces a special kind of diffraction photograph, very unlike the original. The likeness is restored in a second process, carried out with light, in which the photograph is used as the diffracting object. The essential feature of both diffraction processes is, *coherent illumination, and a strong coherent background.* The criterion of coherence, and its practical consequences will be discussed later. For the moment it will be sufficient to assume an *absolutely coherent* illuminating beam, such as could be produced, theoretically, by a monochromatic point source. Assuming such a source it is permissible to introduce between the point source and the object optical systems with geometrical errors, as a lens, however bad, does not destroy the coherence [1].

In the optical experiments the object was a micrograph and the arrangement was the same as if one wanted to cast an enlarged shadow

of the object on a screen. But the finite wavelength of light makes the "physical shadow" very different from the geometrical shadow, sometimes quite unrecognizeable, as will be seen later.

In the second step, which may be called "synthesis", the object is removed, and the photograph or "hologram", as it is callied, is illuminated by the same beam, though not necessarily with the same wavelength. If now one places a good lens behind the hologram, large enough to cover it, and looks through this lens towards the source, an image of the object will be seen, as if it were in position. As it is rather difficult to obtain lenses free enough from smal defects, which would pass unnoticed in ordinary microscopy, but become very troublesome in coherent illumination, first experiments were made without a lens and with a pinhole of about 5 microns. The resolving power obtained in these samples agrees very well with what one calculates by Abbe's formula from the wavelength and the divergence of the illuminating beam.

Direct pinhole illumination cannot be applied in the electronic scheme, as it is impossible to produce pinholes of the order of the resolving power, *i.e.* a few A.U. It is necessary to start from a larger pinhole and to reduce it by lenses. This of course means introducing geometrical errors, spherical aberration and astigmatism, and it is the most important feature of diffraction microscopy that these do not affect the obtainable resolution, so long as *the geometrical errors are the same in the analysis as in the synthesis*. To test this result of the theory we had to use lenses with extreme aberrations. This was achieved by turning the microscope objective the wrong way, and enlarging its aberrations by a second lens, *i.e.* carefully breaking all rules of sound optical design. Thus we obtained a caustic which was 6 mm long at an aperture of less than 0.1, and a cross section of minimum confusion of about 100 microns diameter. (Fig. 1). A lens

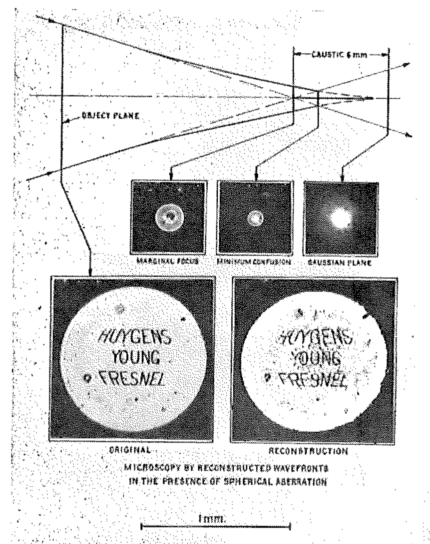

Fig. 1.

of such bad quality, used as an objective could hardly reveal details finer than 50 microns, yet the reconstruction proves that the overall resolution was of the order of 10 microns or better. This is the most direct proof of the principle of reconstruction yet obtained. In fact it was rather difficult to produce these photographs, but the reasons were of a secondary nature. The objectives were full of local imperfections, and these produced a very uneven background on the photographic plate, full of false detail. In order to get at the true detail it was necessary to subtract the background photographically, as illustrated in Fig. 2.

Even so some "noise" persists in the background. It is comforting to think that we shall not have to contend with this difficulty in the electronic scheme. However bad an electron lens is, it is always perfectly polished, it has neither scratches, dust or cementing specks.

Thus the optical model experiments have satisfactorily confirmed

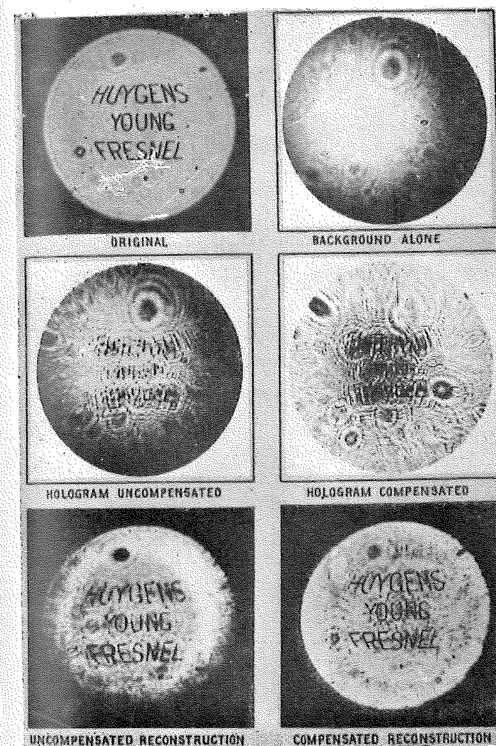

Fig. 2.

the theoretical expectations, and we have a fairly solid foundation to undertake the development of the electronic-optical scheme.

The chief difficulty of the electronic part of the apparatus arises directly from the principle, which requires a *strong coherent background*. It will be now necessary to define what we mean by sufficient coherence. Broadly speaking this means that the interference fringes must not be appreciably washed out near the periphery of the hologram, which corresponds to the useful cone of illumination, with a semi-angle α. This divergence angle is determined by Abbe's relation $d = \frac{1}{2}\lambda/\sin\alpha$ *i.e.* it must be wide enough to produce the desired resolution limit *d*. Satisfactory coherence up to this divergence angle is guaranteed if two criteria are satisfied. The first concerns the monochromatism of the beam, which must be spectrally pure to the degree of allowing interferences up to a certain maximum order. In the optical experiments we had not the slightest difficulty in satisfying this criterion, even the relatively broad lines of the high pressure mercury arc used produced good fringes to the order of a hundred or even more. Monochromatism of the order of one in a few hundred appears a very easy matter in electron optics, commercial electron microscopes operate with variations of the de Broglie wavelength of 1 : 10,000 and even 1 : 100,000. Yet a considerable difficulty arises in the electronic application, to which we will return in a moment.

The second criterion of coherence concerns the size of the source. One can formulate it very simply in the form that the "incoherent diameter" of the source must be about equal to the Abbe limit of resolution. This corresponds to about 70 % coherence at the edge of the hologram in Zernike's formulation of the degree of coherence [2]. One can apply this criterion directly to the pinhole sources used in the optical experiments. If the pinhole is imaged by a

further system, the criterion must be extended by stating that *the nominal or Gaussian diameter of the image of the pinhole must be smaller than the resolution limit d*. It is of course the very foundation of the method of diffraction microscopy that the physical diameter of the beam can be much larger, 1000 times or more. In fact if we want a resolving power ten times better than the present, and use the best available objectives as condensers for the illuminating beam, we shall have to open up the aperture ten times, which means that the minimum diameter of the beam increases 1000 times.

The small size of the source compatible with coherence is a first difficulty of the electronic scheme. One can work out that the coherent current is only of the order of a few 10^{-14} amps. Note that the coherent current is quite independent of the resolving power which one wants to achieve, but for good resolution one will require of course a larger photographic plate. For tungsten cathodes, fine grain plates and resolving powers of the order of 1 A.U. one thus arrives at exposures of the order of one hour. This is an inconveniently long exposure, though not prohibitive. It is very natural to think of reducing it by using emitters with very high densities, in particular autoelectronic emitters, which, according to the researches of Benjamin and Jenkins [3, 4] can be expected to show a reasonably constant behaviour at such small currents, (less than 10^{-10} amps.) and to yield current densities at least 1000 times larger. This would reduce the exposure to seconds, instead of hours, but before we can think of applying them, we must solve a problem arising from the required monochromatism of the beam.

It has been mentioned that no difficulty due to insufficient monochromatism was experienced in the optical experiments. In this case a very moderate degree of monochromatism is sufficient, of the order of one part in a few hundred. We should have no difficulty in the electronic scheme either if we could scale down our condenser system in the ratio of the wavelengths of light to electrons, about 1 : 100,000, which is obviously impossible. Thus, measured in wavelengths, the axial length of the chromatic figure will be enormously larger in the electronic apparatus than it was in the optical model. So in order to make really significant progress, say a step of 10 in resolving power, we require some means for monochromatizing the electron beam, perhaps by a factor of 100 better than at present, or else we must avoid this necessity by using either an optical system between the object and the photographic plate, as proposed by Mr Dyson of A.E.I., Aldermaston, or mirrors which probably must be used asymmetrical. It is true that this raises the formidable theoretical difficulties of non-centered optical systems, but I think the prospects justify undertaking these complicated investigations. I want to mention however that these problems will not become acute for some time, the first steps, up to, and perhaps somewhat beyond the present performance of ordinary electron microscopes will be probably possible with orthodox electron emitters, and with beams monochromatic to the extent as practised at present. Nor must it be thought that if for a start we just attain the resolving power of the orthodox microscope, we shall have photographs just like at present. It is well known that in the case of organic materials the resolving power fails at a level far below the nominal power of the microscope, for sheer lack of contrast. Diffraction microscopy, chiefly by reason of coherence, can explore *phase contrast* to its full extent, and it is known, chiefly by the theoretical investigations of H. Börsch [5], that the phase contrast method promises a very superior resolution of thin structures of low atomic weight. Finally I want to mention that once a hologram is obtained, it can be explored just like a real object, by making the optical synthetizer either an ordinary, or a Zernike-microscope. Thus it can be hoped, that the diffraction principle will allow us to shift the difficulties of phase contrast, like others, from electron optics to light optics, on to the shoulders of the optician, who will be well able to cope with them.

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APPLICATION DES SONDES ELECTRONIQUES A L'ANALYSE METALLOGRAPHIQUE.

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Les sondes électroniques ont été surtout utilisées jusqu'à présent pour l'examen des surfaces à l'aide des électrons secondaires émis, ou bien pour l'étude de la transmission des électrons à travers une couche mince de substance. Or, l'impact des électrons sur la surface d'un object donne naissance à un rayonnement X qui est fonction de la composition atomique du volume irradié. Nous avons cherché à réaliser des analyses chimiques ponctuelles en mesurant et analysant ce rayonnement X.

Cette méthode peut sembler à première vue devoir permettre pour l'analyse une localisation extrêmement poussée. On a en effet réalisé des sondes dont le diamètre descendait à 200 U.A. environ. Malheureusement, des considérations d'intensité et de diffusion des électrons dans la matière rendent inutile, comme nous le verrons tout à l'heure, l'emploi de sondes inférieures à 1 micron.

Nous avons donc cherché à obtenir une sonde de cet ordre, transportant un courant d'intensité maxima, et nous avons dans ce but réalisé un appareil expérimental par transformation d'un microscope électrostatique de la Compagnie Française de Télégraphie sans fil.

Le montage est représenté schématiquement Fig. 1. La première lentille réductrice est constituée par l'objectif du microscope. Sa distance focale peut varier de 6,5 à 40 mm.

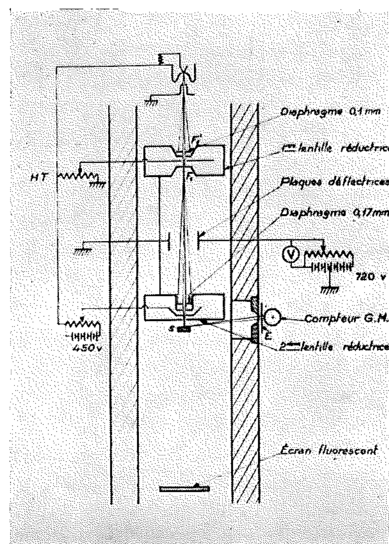

Fig. 1.

environ sous l'action d'un potentiomètre constitué par une simple tige de bakélite. On obtient ainsi au foyer de cette lentille une image du cross-over dont le diamètre varie de 7 à 40 μ environ. Cette image est réduite dans le rapport fixe de 1/20 par une seconde lentille de forme dissymétrique construite au laboratoire. La forme de cette lentille est imposée par la nécessité où l'on se trouve de placer l'échantillon à l'extérieur de la lentille de façon à pouvoir capter le rayonnement X émis. Sa distance focale est de 15 mm. environ et son foyer image se trouve à 5 mm. au dessous de sa face inférieure. L'échantillon peut être déplacé mécaniquement dans deux directions perpendiculaires à l'axe du faisceau. Deux paires de plaques déflectrices permettent en outre d'imprimer à la sonde de très petits déplacements dont l'amplitude maxima est de 50 μ environ. L'objet étant un échantillon métallographique opaque aux électrons, la mise au point de la sonde s'effectue sur une arête métallique tranchante placée à la même hauteur que la surface à étudier, et dont l'ombre est observée sur l'écran fluorescent du microscope.

Les rayons X émis par l'objet sortent sous un angle d'émergence de 6° environ, et quittent le microscope en traversant une fenêtre constituée par une feuille d'aluminium de 0,01 mm. d'épaisseur.

Nous avons tout d'abord essayé de déterminer le diamètre réel de la sonde obtenue en cherchant à l'occulter par des fils métalliques de diamètre décroissant, dont l'ombre était observée sur l'écran fluorescent. Ces fils étaient en réalité constitués par l'extrémité d'aiguilles ordinaires amincies par polissage électrolytique. On peut ainsi obtenir à l'extrémité de l'aiguille une partie cylindrique dont le diamètre peut descendre à 1 micron environ.

Nous avons ainsi pu vérifier que le diamètre minimum de la sonde était de l'ordre du micron, le débit électronique étant alors voisin de 10^{-9} A. Par variation de la distance focale de la première lentille réductrice, le diamètre réel de la sonde augmente en même temps que son intensité. Nous avons choisi pour nos essais une sonde de diamètre 1,5 μ environ transportant un courant de 4.10^{-9} A. pour une tension accélératrice de 30 kV environ, valeur que l'on ne doit pas dépasser comme nous le verrons tout à l'heure. L'intensité théorique maxima que l'on peut atteindre sur une sonde de 1,5 μ de diamètre avec une lentille dont le coefficient d'aberration sphérique Cf est égal à 20 cm et un canon à électrons parfait est d'environ 2.10^{-7} A.; le rendement est donc de l'ordre de 2 %. Le rendement du canon étant de l'ordre de 7 %, la perte est due à l'astigmatisme de la seconde lentille réductrice. Le défaut de blindage magnétique dû à la position du foyer hors de la lentille est en grande partie responsable de cet astigmatisme.

Cette intensité électronique relativement faible rend difficile l'analyse du rayonnement X par les méthodes usuelles de spectographie; plusieurs méthodes restent cependant pos-

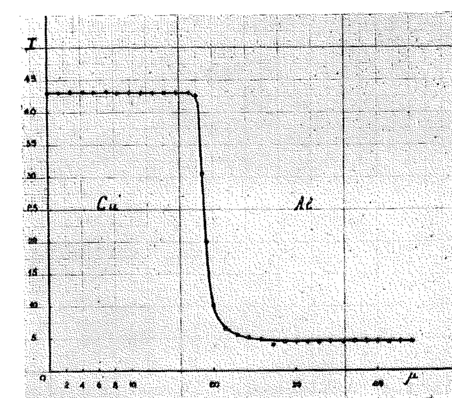

Fig. 2.

sibles dans lesquelles un faisceau de grand angle solide est reçu sur un compteur de Geiger-Müller.

Tout d'abord, la différenciation entre un élément lourd et un élément léger peut s'opérer aisément par la simple mesure de l'intensité totale du rayonnement. Les éléments légers donnent en effet un spectre continu très faible et des raies très absorbables. Il en résulte que l'intensité totale après traversée de deux fenêtres en aluminium est très faible. Les éléments lourds au contraire, donnant un spectre continu intense et des raies pénétrantes, donnent naissance à une forte intensité. La Fig. 2 représente la courbe obtenue par analyse d'un échantillon constitué par un bloc de cuivre et un bloc d'aluminium accolés. On peut voir que l'intensité est dans le cas de l'aluminium plus de 10 fois plus faible que dans le cas du cuivre.

La Fig. 3 représente la courbe obtenue par analyse d'une fonte contenant des inclusions de graphite en forme de filaments de un à quelques microns d'épaisseur. Les décroissances brusques de l'intensité correspondent au passage de la sonde sur les inclusions de graphite. Le carbone ayant un nombre atomique très faible, l'émission du graphite est pratiquement nulle; on observe cependant une intensité encore importante sur les inclusions dont le diamètre est de l'ordre du micron; cela tient à ce qu'une épaisseur aussi faible de graphite est impuissante à arrêter les électrons qui atteignent le fer sous-jacent; il y a là une limitation dans la finesse de la méthode du côté des éléments légers.

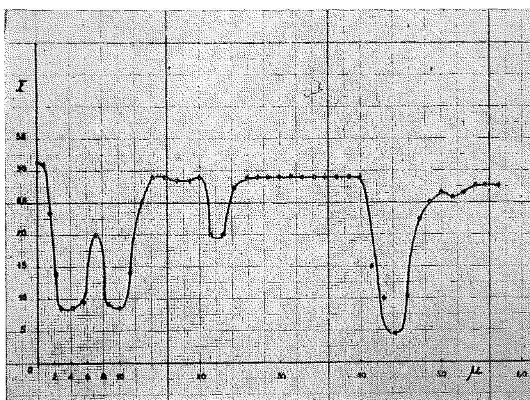

Fig. 3.

Pour les éléments de nombres atomiques voisins, l'intensité des raies caractéristiques Kα peut être mesurée sans spectrographe par la méthode des doubles filtres de Ross. Cette méthode permet l'analyse de rayonnements de grand angle solide, et par suite l'utilisation d'intensités électroniques incidentes très faibles.

Signalons enfin que des essais de mesure de l'intensité des rayonnements Kα au moyen d'un spectrographe à crystal courbé utilisant comme récepteur un compteur Geiger-Müller ont donné des résultats tout à fait satisfaisants.

Il nous reste à voir quelle est la finesse de l'analyse obtenue, c'est-à-dire la valeur minima du volume de matière analysée. Ce volume est déterminé par la diffusion des électrons dans la matière et le calcul théorique en est pratiquement impossible.

Les résultats expérimentaux sur la pénétration diffuse des électrons dans les différents métaux semblent montrer que pour des métaux de nombre atomique moyen et une tension accélératrice de 30 kV, l'intensité électronique est réduite à une valeur négligeable au bout d'un parcours de 2 μ environ. Si l'on admet que ce parcours à lieu également dans toutes des directions, on trouve pour la région analysée un diamètre minimum de 4 μ et une profondeur de

2 μ. L'expérience semble cependant donner des résultats plus encourageants.

Si nous considérons (Fig. 2) la courbe obtenue avec le placage aluminium-cuivre nous pouvons remarquer que la séparation entre le cuivre et l'aluminium se fait pratiquement pour un déplacement de la sonde de 3,5 μ. Le diamètre de la sonde était dans l'expérience de 1,5 μ; il en résulte que l'élargissement dû à la diffusion n'est que de 2 μ environ; le cas est cependant assez défavorable, l'aluminium étant un métal léger.

La diffusion peut être réduite notablement par l'emploi de tensions accélératrices faibles, mais on est limité dans cette voie par la décroissance rapide de l'intensité du rayonnement X. Il faut donc réaliser un compromis entre des qualités difficilement compatibles de sensibilité et de pouvoir de résolution. L'étude se poursuit actuellement dans le but de préciser les diverses conditions. Les résultats déjà acquis permettent de compter sur un diamètre de 3 à 4 μ et une profondeur de 2 μ pour la région analysée. Soit un volume de 20 μ^3 et une massa analysée de l'ordre de 10^{-10} g.

Le gain est déjà d'un facteur 5000 sur la spectrographie ponctuelle et justifie amplement la poursuite des recherches dans cette voie.

Discussion.

Prof. Gabor: If I understand well the volume discrimination could not be made better even if the electron-optical limitations could be overcome, as diffusion limits it to about 1 micron cube?

Réponse: C'est absolument exact.

Ir Le Poole: Is the current 10^{-9} A. the highest possible?

Dr Oosterkamp: The current mentioned, 10^{-9} A. for a focal spot of 1μ, is far below thermal limits. Is it possible by other electron-optical means to obtain a higher current without increasing the size of the focal spot?

Réponse: La limite théorique est d'environ 2. 10^{-7} A. pour des lentilles électrostatistiques. L'emploi de lentilles magnétiques pourrait permettre d'élever cette limite théorique à 4. 10^{-6} A.

Prof. Goldsztaub: La cratérisation observée sur les anticathodes est due en grande partie aux gaz occlus. Un dégazage par fusion sous vide la réduit considérablement.

Réponse: Dans nos expériences, l'intensité électronique était beaucoup trop faible pour donner naissance à des effets thermiques appréciables.

Mr Beekhuis: Is the sensitivity of measuring sufficient to determine the different intermetallic compounds and the slight variations in the composition of them?

Réponse: La sensibilité de la méthode varie en raison inverse de son pouvoir de résolution. Son étude est en cours à l'heure actuelle.

MAGNIFICATION CALIBRATION: THE USE OF LATEX PARTICLES AND ALTERNATIVE METHODS.

V. E. COSSLETT, Cavendish Laboratory, Cambridge, Eng.

1. It was announced by Backus and Williams[1], that a particular batch (580 G) of Latex suspension, made by the Dow Chemical Company, contained particles of remarkable regularity of size. The end result of careful measurement in the electron microscope, calibrated directly against a replica of an optical diffraction grating, gave a particle size of 2590 ± 25 A.U., according to them. They kindly made available samples of the suspension for the use of other workers, and we used some to check the magnification of our Siemens instrument. A discrepancy of 12 % appeared, which led us to make a careful comparison with other methods of magnification calibration.

2. The standard method of calibration employed in the Siemens electron microscope is the measurement of the magnification in each stage, using apertures of known size. In the first stage, an aperture of 70 microns is used as object and the image is measured on the intermediate screen. In the second stage, the diameter of the field of view in the final image is measured, as defined by an aperture of about 1 mm. diameter inserted in the projector pole-piece. Suitable correction must be made for the position of the measured intermediate image being above the focal plane of the projector lens. The standard apertures can be measured with sufficient accuracy (1 %) with an optical microscope. The chief error of the method is in measuring the size of the image on the intermediate screen. This could be photographed, but we had simply observed it directly with a mm. scale. Therefore we could not be sure of our calibration, against the apparent size of the Latex particles, and we proceeded to make an independent check.

3. The diatom, *Synedra fulgens,* shows a large number of parallel rows of holes with a spacing of about 0.66 micron. The spacing varies by a few percent along a specimen, and among individuals. The size is still not too small for the average spacing to be measured with an optical microscope, but we could not be sure enough of the magnification in such circumstances. A photograph was therefore taken at lower magnification in a Vickers projection microscope, of a diatom about 100 microns long stretching across a complete grid square and resting on a collodion film carrying Latex particles; the magnification was measured to

be 242.5 ± 1.25. The same square was photographed in the electron microscope both as a whole at a magnification of 600 x, and in two sections at 1500 x. These two electron optical magnifications could thus be checked directly, to better than 1 %; the value obtained was the average value across the field, without taking account of distortion.

The magnification thus obtained agreed to within 1 % with the calibration by the two-stage method. The average row spacing of the diatom (over 100 rows) was 0.645 micron, falling within the range of 0.63 — 0.69 micron found optically by Dr N. Ingram Hendey, of the Admiralty Central Metallurgical Laboratory, who kindly supplied the specimens used. It thus appeared that the Latex particles must be smaller than assumed.

4. The size of the Latex particles was then measured in two ways; from enlargements of the x 1500 photographs, and from micrographs taken at higher magnification (7000 x), the value of which was checked by direct comparison of the relative separations of characteristic rows of holes in the diatom. This check is safer than measuring between groups of particles, since the collodion may shrink whereas the diatom is very resistant to beam effects. By measuring a large number of particles a size distribution curve was obtained. The value of 112 individuals was 2300 ± 50 A.U. Correction for distortion of the image would have given a rather smaller spread, but would not significantly affect the mean value.

5. Similar results have been obtained by Scott[2], who finds in addition that the size obtained depends on the exact conditions of drying down the Latex on the supporting film, varying between 2500 and 3000 A.U. It may also depend on the age of the sample, and the effect of the vacuum and electron beam, in our opinion. Williams observed that the size of metal-shadowed particles was about 10 % less than that of the shadows. He ascribed it to the effect of long range Van der Waals forces in deflecting passing electrons. It seems more likely to be due to deflection of charged metal atoms by normal Coulomb forces in the shadowing process, the Latex being charged by induction; they are of insulating material and on an insulating support. The removal of tightly bound water under impact of the electron beam may also contribute. Continued exposure to the beam resulted in visible shrinkage, even to elliptical form. A very strong beam also caused aggregation of the shadowing material (gold-palladium) into a definite "pip", indicating the possible existence of irregularities on the surface of the Latex particle.

6. It must be concluded that the Latex particles are unsuitable as an absolute standard of calibration, both on account of the variation in particle size and their change under the beam. The two-stage method, or reference to a standard diatom, is preferable but more cumbersome; an accuracy of the order of 1 % could then be assured. Three-stage microscopes would have to rely on the diatom. The Latex particles are very useful for mixing with specimens, so long as an accuracy of no better than 5—10 % is required.

References.

1. Backus and Williams, J. Appl. Phys., 19 (1948) 1186.
2. Scott, J. Appl. Phys., 20 (1949) 417.

Discussion.

Two confirmations of the variation in size were quoted, and one of William's figures (by Mr Farrant). Mr Haine suggested the sharpness of the shadow edges might allow a decision between Coulomb or Van der Waals forces as the origin of the smaller size of the particles. Dr Locquin proposed the use of particles of calcium carbonate, which are very uniform when produced in the metabolism of myxomycetes (see p. 150). Ir Induni employed a directly calibrated stage-shift for magnification measurement in the Trüb-Täuber electron microscope. The accuracy is at present 10 % and should be capable of improvement.

RESOLVING POWER.

M. E. HAINE, Associated Electrical Industries Research Laboratory,
Aldermaston, Berks.

I suppose this is one of the most controversial aspects of microscopy. The best one can do in discussing it is to try and point out some of the outstanding difficulties and misapprehensions which give rise to controversy.

A special committee of the Electron Microscope Society of America have considered some aspects of the subject and a report on their deliberations is given in a paper in J. Appl. Phys., December 1946. In particular, this committee have appreciated the difficulty due to nomenclature. Resolving power has been and is still defined by many as the power of the microscope to resolve two closely spaced point sources of illumination so that they may be recognised as distinct. This idea of the power to separate two points or two lines, *etc.*, came about as a result of Abbe's diffraction grating theories. It is useful in talking about a microgram, but apt to be confusing in talking about the resolving power of the instrument. It is convenient to separate the resolving power of the instrument from the resolution of the picture. The committee pointed out this distinction and, in general agreement, I would like to repeat their suggested definitions and urge that they be accepted by the rest of us.

Resolution is a function of the micrograph and specifies its sharpness. It is inevitably largely qualitative in nature and could only be made quantitative if further specified, for the sharpness may vary by large factors over a picture depending on the type of detail and also to a certain extent on the contrast conditions. It depends, of course, on the instrument performance and in certain specific cases can be correlated to this as we shall see. It is also important to realise, that photographic technique can vitally affect the micrograph resolution. Not only to the extent of worsening by bad focusing *etc.*, but also to the extent of considerably improving it as judged by sharpness of edges, for example. The latter, of course, is not a true improvement although one hears it argued as such.

Resolving power, on the other hand, is an instrumental factor and defines the capability of the optical system to produce a true image. Now here I differ slightly from the Committee's suggestions. I feel the term resolving power should define the resolution which the instrument

should, or does give in its final image under prescribed conditions, *i.e.* of illumination, specimen, *etc.* The *resolving limit* or resolution limit is then defined as the best resolving power of the instrument under ideally specified object conditions.

We then see that the resolving limit which may be further specified as the practical, theoretical or ultimate resolving limit, describes essentially the quality of the optical system, while the resolving power describes how a given object detail will be imaged.

By thus deciding upon suitable definitions, we can avoid a certain amount of unnecessary controversy, but the biggest problem is still undecided; namely, on what measurements do we base our definitions. Here a difficulty arises, mainly due to the fact that there are two logical methods of approaching the problem. On the one hand, imagery in a lens system is treated by the optician on a point by point basis. That is to say the images of single points are computed and integrated to give the total image, taking into account where necessary the question of coherence between the point images. On the other hand, coherence may be brought in at an earlier stage and the imagery treated as a diffraction problem from scratch, the object being a more or less complicated diffraction grating.

Both these methods are sound if correctly treated, and their equivalence forms the basis of the famous Abbe controversy.

The former approach is to be preferred in considering resolving limit. If we accept this then we base our definitions not on separation of lines or particles but on the imagery of a single point source.

Having deduced or measured the image pattern due to the point object, however, we must still be careful not to misapply the result in interpretation of the complete image.

The image of a point source by a microscope objective system has been discussed by many authors. The image intensity distribution is dependent mutually on the diffraction effects produced by the effective objective aperture and by the aberrations of the system. With no aberrations the distribution is the Airy pattern.

This distribution may be arrived at by considering a spherical wave leaving the object point, expanding radially outward and at the lens being inverted to a spherical wavefront centred at the Gaussian image point, and limited in lateral extent by the objective aperture. If now this wavefront is considered as a Huygens radiator of spherical wavelets, all starting from the wavefront with equal phase, integration of the wavelet amplitudes at points across the Gaussian image plane, taking into account the appropriate phases and squaring, gives the required energy distribution.

In the presence of spherical aberration the original spherical wavefront is now inverted to a position differing from the spherical by an amount $C\,\alpha^{\frac{1}{4}}$. Integration then gives a distribution quite similar to the Airy curve. Angular variation in electron density leaving the object can be accounted for by variation in the wavelet amplitudes across the aspheric wavefront.

An approximate connection between the radius of the confusion disc with and without spherical aberration was suggested by von Ardenne:

$$\Delta_t = \sqrt{\left(\frac{0.61\,\lambda}{\alpha}\right)^2 + (C_s\,\alpha^3)^2}.$$

Δ_t is here the confusion disc radius for a wavelength λ, semi-aperture angle α and spherical constant C_s

Δ_t will have a minimum value for some optimum values of α:

$$\Delta_{t\ opt} = 0.95\,\lambda \sqrt[4]{\frac{C_s}{\lambda}}$$

(This is only 14 % larger than the Airy disc for the same aperture).

For $C_s = 1$ cm. and $\lambda = .05$ A.U.

$$\underline{\underline{\Delta_{t\ opt} = 10 \text{ A.U.s.}}}$$

It is worth bearing in mind that many alternative figures quoted are merely related to a different measurement, such as the half width of the Airy disc, *etc. etc.*

Having then a knowledge of the intensity distribution in the image of a point object, we can compare this with that obtained in practice and judge the degree of perfection of our instrument, provided we can find a suitable object.

The next and very important step is to see if we can get some insight into the relation between the known point image pattern and that for objects of finite size and definite shape. Here we run into difficulty. In principle we can proceed and evaluate the image contrast variations for any object, but in practice the procedure can be very complicated.

If we can assume that the radiation from the object is completely incoherent and is emitted with constant amplitude into the whole of the objective aperture, very great simplification results. The image intensity distribution is then obtained by arithmetic summation of all the overlapping point images in the image plane. It is only necessary to know the relative magnitude of these, that is to evaluate the contrast point by point from the object and carry out the integration. This integration can be achieved by simple semi-graphical methods. For example, the integrated patterns for edges, slits, discs and holes are simply evaluated.

Knowing the theoretical relations between such object details and the point image distribution, it should be possible to deduce the point image from a picture of such detail. We then have the big advantage that, for instance, in the case of the straight edge a suitable object can be prepared relatively easily, whereas to obtain a point object requires a particle which is small compared with the resolution limit and, as is well known, such a particle will in general give negligible contrast.

Unfortunately, our assumptions are not in general admissible. In particular we do not as a rule meet the requirement of incoherence of the radiation from the object. We can review in a general way the implications of this. If an object is self-radiating, every point emits radiation bearing a random phase relationship with every other. Thus, in an image the total distribution of intensity is the sum of the intensity distribution due to each object point, as before described.

If, however, the object is illuminated from a source which is not ideally focused on the object and the object points are not ideally diffusive in nature, then the total intensity can only be obtained by the complicated process of integrating vectorily at each point the amplitudes of all the wavelets incident there,

taking due account of their phase. The intensity is then the square of the integrated amplitudes. This process is, in general, very laborious and not of general use in our day-to-day work.

Coherent illumination would generally appear to increase the effective confusion disc. On the other hand, it can be observed that a slightly out-of-focus edge with a pronounced Fresnel edge pattern will give every appearance of being much sharper than the in-focus edge. This effect can lead to very misleading estimates of resolving power. The effect would appear to be due to the increased contrast ratio at the edge which may be two or three, or even more, times that between points well inside and outside the edge. The effect can easily be greatly enhanced if insufficient care is taken in the photographic recording.

The measurement of resolving power in the electron microscope with any degree of accuracy or certainty at present then appears impossible. The degree of coherence in the irradiating beam can never be reduced sufficiently to be neglected. Indeed, even if so called critical focusing is used, the coherence is still sufficient to destroy the intensity minimum between the images of two point particles separated by the Airy disc radius. The most promising test would appear to be a very small aperture in an opaque disc. This will give a fairly accurate Airy disc and observable contrast, but such an object is physically not realisable.

We are left then with the alternative of deducing a figure for resolving power using object conditions only roughly approximating to the ideal, having reasonable assurance that the figure obtained will, in general, be a pessimistic one unless we distort the object contrast by inaccurate photography. Incorrect deduction of resolving power from the appearance of the microgram is all too common. One example can be quoted as illustrative of wrong application of the physical concepts. The example refers to optical microscopy. The operator observes an object which contains a relatively transparent slit between two less transparent regions. He knows from electron microscope observations that the width of the slit is 1,000 A.U. He finds in his optical microscope that a clearly seen maximum of intensity appears in the region of the slit. Therefore, he says the slit is resolved and the microscope resolution is 1,000 A.U. The error, of course, arises from the fact that a slit between opaque edges will transmit some energy, however narrow, and therefore will give some contrast. The resolution should not be determined from the known slit width but by the width of the contrast maximum which is actually twice the Airy disc radius, plus the gap width.

Again we may quote an example concerning coherent radiation: Can the recording of a Fresnel pattern with a spacing of 10 A.U. be interpreted as a resolution of this order? If one imagines the objective lens as consisting of two zones, axial and marginal, each of which produces an image of the Fresnel pattern in each of two object planes, the superposition of the two coherent images can still give a pattern even if the separate images are displaced by a half fringe. This suggests that a better resolution than the true one may be indicated.

In general, it is seen that the measurement of resolving power or resolution limit is at least as difficult as in the optical microscope. It appears strongly desirable to borrow experience from the optical field and, at least for the present, concentrate on finding an object which can perform the same function as the Diatom which will act as a standard object for electron microscope performance. Such an object might be a recrystallised metallic film if a reproducible method of preparation could be devised.

In conclusion, I feel one ought to briefly review the present prospects of improving on the resolving limit of our instruments. I think we can fairly say that practically all instruments in operation are limited before their theoretical limit by mechanical or electrical errors. The main cause being objective lens pole piece asymmetry or ellipticity. This problem, however, has been investigated by several groups, and I think we can say the solution is now known, or is at least in sight. The effect is minimised by the use of wide bore pole pieces and can effectively be corrected by field compensation as done by Hillier and later by others.

It would appear then that there is a reasonable chance of obtaining commercial instruments with a true resolving limit of 10—20 A.U. as judged by the radius of the point diffraction pattern. The resolution obtained with these instruments still depends on specimen conditions to a large extent of course.

Further improvement requires methods of reducing spherical aberration, and having found these of re-establishing the contrast mechanism which would largely be lost if wider apertures were used, though here chromatic scattering and aberration may provide an adequate answer.

Methods of reducing spherical aberration by space charge and axial electrode systems have been suggested by Gabor. The methods do not appear very practical at present. Scherzer has suggested using a lens with one electrode in the form of a thin foil through which the electrons must penetrate, and also the use of crossed cylindrical lenses. These methods appear more hopeful, though the first would probably require higher than usual beam energies. The second method is complicated to set up but appears not impractical.

Gabor's diffraction microscope of which we have heard is a completely new idea and has great promise, though it is somewhat restricted in its direct application. The brilliant conception of the diffraction microscope may have far reaching results in time to come, and we should thank the author for the great inspiration he gives to us and to our subject.

Discussion.

Prof. Gabor: May I suggest an International Working Committee to make proposals for Internationally acceptable Standards on resolution. The mathematical work does not appear very difficult in view of the optical work of Maréchal in France and Hopkins in England. But it will be also necessary to include practical people to obtain a concensus of opinion on what will be acceptable to the manufacturers as a basis of judging their instruments.

Answer: I would readily second Professor Gabor's proposal but let me stress one point I have made. The theoretical basis of resolution is one matter and the practical measurement and comparison another. I feel sure we must look, not so much for theoretical interpretation, as for a standard type of test specimen to compare with the diatom in light microscopy for instance. This should be borne in mind in setting up the Working Committee.

Ir Le Rütte: Is it not possible to correct electrostatic lenses for spherical aberration with a mirror. You can use these mirrors under an angle to get the beam out and correct for astigmatism by using a non-rotational electrode.

Answer: The possibility of connecting spherical aberration with electron mirrors

has been put forward by a number of workers on previous occasions. It would be necessary to devise a system to get the image out on a slanting axis, and in general the negative aberrations of the mirror are too large in magnitude to apply directly. A very accurate matching of the negative and positive aberration is, of course, necessary.

Mr Hercock: Is there any limitation by photographic resolution on resolving power of microgram.

Answer: The photographic plate does not, in general, limit the resolution of the instrument. The plate resolution might become very important if fluorescent screens of greater resolving power were available.

IMPROVED INVESTIGATION METHODS FOR ELECTRON LENSES.

G. LIEBMANN, Associated Electrical Industries Research Laboratory, Aldermaston, Berks.

The performance of most electron optical devices is limited by certain aberrations; the most important ones are astigmatism and coma due to ellipticity and decentring of the lens components, and spherical aberration. This last error has, very rightly, received considerable attention during the last 10 or 12 years, but most discussions were based on certain few and highly idealised cases.

We thought that a closer correlation between the geometry of the lens components, the resulting field shape, and the imaging properties of electron lenses was desirable. The problem of designing an electron lens with certain prescribed properties is a frequently recurring one, not only in connection with electron microscopes. Moreover, it seemed that only a closer study of the relation between field shape and aberrations would make progress possible towards improved lenses. We were therefore interested in investigation methods which can be applied to electron lenses of *arbitrarily prescribed geometry* of the lens components. Rigorous mathematical solutions are then not possible, and one has to use either numerical mathematical methods, or experimental methods. We decided to use both these methods, to supplement and to support each other.

The well-known electrolytic tank is liable to considerable errors, and calls for great care in its operation if an accuracy of better than 1 % is required. But the evaluation of lens aberrations by computational methods necessitates an accuracy at least 20 times higher. About two years ago de Packh in America described another analogue, in which the continuous conductive medium of the electrolytic tank is replaced by a network of interconnected resistances. If the resistance values are suitably graded, such arrangement can represent an inclined bottom tank for the investigation of axially symmetrical fields. However, de Packh indicated that the accuracy of the method would be poor, of the order of a few percent, being limited not only by the finite mesh size, but still more so by the tolerances of the resistance values. This seemed confirmed by an apparatus constructed by Redshaw in England; the accuracy of field plots published a short time ago by Redshaw is of the order of 2 % to 3 %. Thus, the

resistor network analogue did not seem to offer any advantage over the electrolytic tank. However, on checking de Packh's work, we took the analysis of the operation of the resistance network a good way further, and came to expect an altogether different degree of accuracy.

The result of our analysis is this: if we assume the resistance tolerance errors $\triangle R/R = \varrho$, and the resulting errors $\delta V/V_0$ in the field plot, to be randomly distributed, such as to produce a "normal" error frequency curve, we can define the errors through their "standard deviations" $\sigma(\varrho)$ and $\sigma(\delta V/V_0)$.

Our theory leads then to this predicted error of the potential distribution at any point (r, z): (1)

$$(1) \quad \sigma(\delta V/V_0) = \frac{4}{5}\,\sigma(\varrho)\left[\left(\frac{E_r}{E_a}\right)^2\left(\frac{h}{a}\right)^2 + \left(\frac{E_z}{E_b}\right)^2\left(\frac{h}{b}\right)^2\right]^{1/2}$$

In this formula, a and b are the maximum dimensions of the model measured in mesh lengths h, and E_r and E_z are the field gradients in the r and z directions. E_a and E_b are the average field values in the r and z directions, and V_0 is the applied voltage.

The resistor network which we subsequently constructed, and which has now been in operation for nearly a year, employs 60 meshes in the z-direction and 20 meshes in the r-direction. From our formula we can then predict that the expected errors in a lens problem will be somewhere between 5 parts in 10,000 and 1 part in 10,000, giving us the accuracy which we had desired, although the tolerances of the resistances used are as high as ± 1 %.

An experimental confirmation of these predictions is shown in Fig. 1,

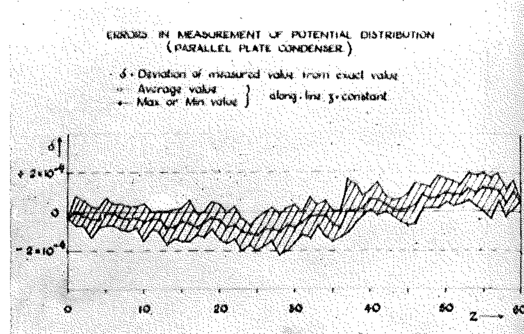

Fig. 1.

where the deviations of the actual measured potential values from exact values are plotted for the simple case of an infinitely large parallel plate condenser, the plates occupying the positions z = 0 and z = 60. The central line, $\delta V = 0$, corresponds to the exact value V (r, z) = z/60, the dashed lines the limits ± 2 parts in 10,000. The circled central line represents the deviations of the *average* potential values from the exact values for the respective z-coordinates, and the upper and lower full lines the *maximum* errors occurring at the respective z-coordinate. Our formula would give a value of $\sigma(\delta V) = 6.7 \times 10^{-5}$; this means a "probable error" of 4.5×10^{-5}, and would predict 4 errors greater than 2.0×10^{-4} and practically none outside $\pm 2.7 \times 10^{-4}$; the experimental result is 3 errors amongst 1239 mea-

suring points greater than 2.0×10^{-4}, and none outside 2.2×10^{-4}; this seems a very reasonable agreement, which has been confirmed in many other cases.

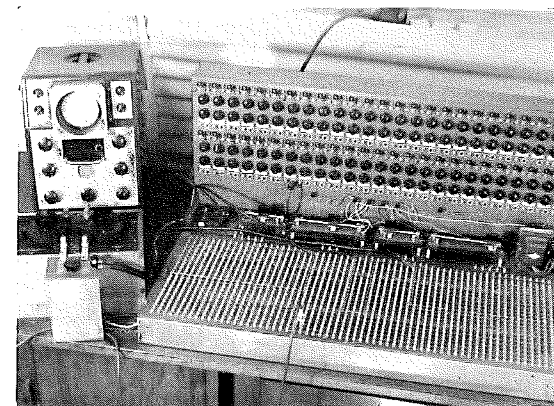

Fig. 2.

Fig. 2 shows a photograph of the apparatus. The resistor units are 1 watt high stability carbon resistors, varying in value from 16,000 Ohm near the axis to 100 Ohms at r = 20 units, being graded logarithmically in the r-direction. The resistor units are attached to studs screwed into a bakelite panel; all connections are made by soldering. To set up the desired boundary conditions, a heavy gauge tinned copper wire is run along the appropriate studs. The apparatus is mostly fed with 6.5 volts A.C. (50 c.p.s.); two other transformer voltages are available if needed and two intermediate adjustable voltages can be tapped off two potentiometers. The potentials are measured with a probe. The result is given directly as fraction of the total applied voltage, using a precision potentiometer bridge and an amplifier and C.R.O. as indicator. The readings can be taken to 1 part in 100,000 at full sensitivity. Notable features of the apparatus are its freedom from stray capacity effects and the absence of a high impedance in the probe connection; hence no Wagner earthing bridge is needed.

The apparatus is not restricted to lens models in which the boundaries coincide with the straight lines through the mesh points; any intermediate or *curved* boundary can be easily represented by shunting the resistor connecting the two studs between which the boundary passes by an appropriate externally connected resistor.

It is also possible to measure the potential *within* a mesh to a position accuracy of 0.02 mesh length with the help of a clip-on potentiometer bridge.

The potentialities of the new apparatus are going much further than mentioned so far. As described, it solves automatically Laplace's equation $\nabla^2 V = 0$. However, by applying an iteration process, more general

partial differential equations can be solved; amongst these is the space charge equation, which in normalised units reads $\nabla^2 V = {}^4/_9 \, V^{-1/2}$. In the resistor network we can represent the right hand side of this equation by currents fed in at the mesh points. The actual values of the currents would be given by $I\ (r, z) = K \times V\ (r, z)^{-1/2}$, where K is a known apparatus constant depending on the scale of the model and the resistance values. Now the function $V^{-1/2}$, and therefore the correct currents I, are not known initially, but we can apply this iteration process: 1) we determine the potential distribution $V^0\ (r, z)$ in the absence of space charge; 2) we make $I_0\ (r, z) = K \times V^0\ (r, z)^{-1/2}$, and measure the resulting potential distribution $V_1\ (r, z)$, which is an improvement on $V_0\ (r, z)$; 3) we re-adjust currents to $I_1\ (r, z) = K \times V_1\ (r, z)^{-1/2}$, *etc.* The potential distributions $V_0\ (r, z)$, $V^1\ (r, z)$, $V_2\ (r, z)$, ... converge very quickly towards the correct solution. Thus, the way is open to investigate space charge containing electron lenses.

Having obtained the potential or field distribution with the required high accuracy, we can now proceed to the tracing of the electron trajectories through the lens fields.

The method employed is a further development of the Taylor series method previously used by Maloff and Epstein, and by Cosslett. We use as basis the *general ray equation*

$$r'' = \frac{1 + r'^2}{2\,U} \left| \frac{\delta U}{\delta r} - r' \frac{\delta U}{\delta z} \right| = 0,$$

where $U = \Phi - \dfrac{e}{2\,m} A^2$

is Störmer's "equivalent potential" Φ being the electrostatic potential and A the angular component of the vector potential. We stipulate that the field terms U, $\dfrac{1}{r}\dfrac{\delta U}{\delta r}$ and $\dfrac{\delta U}{\delta z}$ which are functions of r and z, can be represented by a staircase line, *i.e.* are constant for each z-interval. We can then write r_{n+1} as a Taylor series in r_n and the derivatives of r_n, using the differential equation for r'', to express all higher derivatives through r_n and r_n'. If we re-arrange the terms and break off after the first power of r_n and r_n' we obtain the paraxial trajectory. The result can be written as a set of recurrence formulae:

$$r_{n+1} = Q_1 r_n + Q_2 r_n'$$
$$r'_{n+1} = Q_3 r_n' + Q_4 r_n,$$

where the coefficients $Q_1 \ldots Q_4$ are functions of the field values. In the case of a purely magnetic lens, for instance:

$$Q_1 = Q_3 = 1 - k^2 h^2 \frac{\Delta z^2}{2}$$

$$Q_2 = (1 - k^2 h^2 \frac{\Delta z^2}{6}) \, \Delta z$$

$$Q_4 = - k^2 h^2 \, \Delta z,$$

where

$$K^2 = \frac{e\, h_m^2\, R_o^2}{8\, m\, \Phi},$$

and

$$h = h(z) = \frac{H_o(z)}{H_m}.$$

The first order aberrations are obtained by carrying terms to the next higher order of terms in r and r'. The most accurate way to evaluate them found was to determine not the extraparaxial ray itself, but its deviation from the paraxial ray. We then get a similar set of recurrence formulae:

$$\Delta r_{n+1} = Q_1 \Delta r_n + Q_2 \Delta r_n'$$
$$\Delta r'_{n+1} = Q_3 \Delta r_n' + Q_4 \Delta r_n + Q_5,$$

where $Q_1 \ldots Q_4$ are the same as before, and Q_5 contains terms in r_n and r_n' and the derivatives of the field functions.

The usefulness of this way of evaluating the aberrations lies in the possibility of assessing the contributions to the aberrations of particular parts of the lens field without requiring a knowledge of conjugate object and image points, as happens in certain combined lenses, and similar problems.

Having analysed the lens properties in this way, we still wish to make a final experimental check on the result under proper working conditions, and to explore certain effects like iron saturation, or high voltage breakdown. Several methods have been described for the experimental investigation of electron lenses, but none seemed applicable to lenses in which only part of the field is used in the image formation, as is the case in many magnetic electron microscope objectives, one of the most important lens types. In all previous methods, the positions of the incoming and the outgoing ray were determined; bending of the incoming ray in the unused part of the lens field could not be assessed. We developed therefore the method illustrated by Fig. 3. We illuminate a small aperture (the "object") placed on the axis of the lens with a cone of electrons under "critical illumination". The lens images the aperture greatly magnified on to the fluorescent screen. We now place a further aperture behind the lens which selects a small zonal area of the lens. Traversing the lens with this selection aperture permits a study of the focusing properties of the various lens zones. If a photographic plate in the position of the fluorescent screen is moved a little

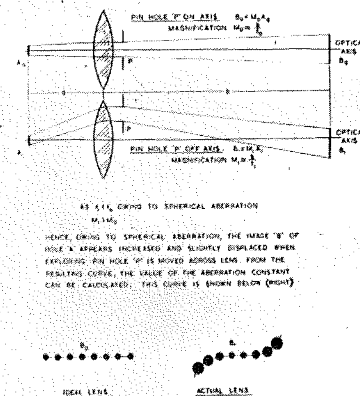

Fig. 3.

each time the zone selection aperture is moved, we can trace out directly a cubic parabola due to the change of zonal focus as a consequence of spherical aberration. The object aperture can be moved along the axis of the lens, and can in particular be placed where a specimen would be located in an electron microscope, regardless of the axial extent of the focusing field. Lens asymmetries can be investigated by rotating the lens with respect to the traversing aperture.

Discussion.

Prof. Grivet: A capacitance-inductance network can be used to determine electron trajectories with about 1 % accuracy. If, according to Prof. Rocard, an amplifier valve is included in each branch of the network, higher accuracy can be obtained and the evaluation of aberrations may be possible.

Answer: One can actually apply a network of pure resistances to the computation of electron trajectories using the iteration process mentioned in the lecture, by treating the electron trajectory as a kind of "eigenvalue" problem.

MECHANICAL INTEGRATOR FOR STUDYING THE DISTRIBUTION OF LIGHT IN THE OPTICAL IMAGE.

A. MARECHAL, Optical Institute, Paris.

(Report presented by Mr A. FRANÇON).

When the geometrical aberrations of an optical or electronic instrument have been determined, it is possible to calculate the surface form of a particular wave Σ in the image space. The Huygens-Fresnel principle then makes possible the calculation of the illumination at a point C in the following manner:

Assuming that S is a sphere with center C, M a point of Σ, I a point where MC intersects S, IM $= \triangle$ is the normal deviation of the wave surface from the sphere S. The components of the amplitude of the vibration are:

$$A = \iint_{T} \cos \psi d\omega, \quad B = \iint_{T} \sin \psi d\omega, (1)$$

where T is the contour of Σ, ψ the phase difference $\psi = 2\pi \triangle / \lambda$ and $d\omega$ the surface element of Σ if the totale surface of Σ is taken as a unit.

The integrator to be described permits the calculation of A and B, and accordingly, the determination of the intensity and phase of the resultant vibration in case of a rotationally symmetrical instrument possessing a circular exit pupil with radius r.

Let us now transform the double integrals into simple integrals using as variables the polar coordinates ϱ and φ with respect to the pupil, we have:

$$d\omega = \frac{1}{\pi R^2} \varrho d\varrho d\varphi \quad (2)$$

Next we describe an Archimedean spiral on that pupil, with a parameter sufficiently small so that we can substitute the integration with relation to the variable ϱ by a finite sum of distant terms of $d\varrho = a$. Then we still have to carry out the continuous integration of the quantities $\varrho \cos \psi$ and $\varrho \sin \psi$ with relation to φ as variable, along the spiral, and it is necessary to determine $\triangle$ (or ψ) as a function of φ. In order to limit the integration to a perfectly circular contour, we have in fact to decrease the width of the spiral at the ends.

The path difference, $\triangle$, depends upon the geometric aberrations and the position of point C; if we define C by cylindrical coordinates with the paraxial focus P as origin, with the axis coinciding with a mean ray (the principal ray), one of the coordinates will be the shift of focus with field given by the distance of C from the plane perpendicular to the axis passing through P; the other two coordinates allow C to vary in the particular focussing plane. It is found

that the path difference is composed of five terms. The addition of these terms may be done mechanically by means of a steel tape passing over five pulleys. The first two terms, which correspond to the spherical aberration and the focusing shift (or the curvature of field), depend only upon ϱ and may be obtained by $\varrho \cos \psi$ and $\varrho \sin \psi$. Therefore two cranks with radius ϱ, (obtained by means of a cam) and turning in proportion to the difference of angular phase ψ, push two integration rollers into contact with the plates whose rotation will be given by ψ. A view of the machine is given on Fig. 1.

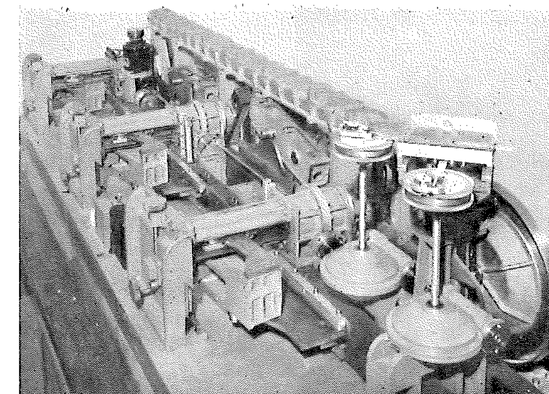

Fig. 1.

means of cams whose displacement represents the variable ϱ (which is a linear function of φ). The term representing the coma of the form $f(\varrho) \cos \varphi$ will be obtained by the movement of a crank turning according to angle φ the radius of which is proportional to $f(\varrho)$ as determined by a cam. The exploration of curvature and astigmatism are represented, respectively, by the terms of the form $\varrho \cos (\varphi - \varphi_0)$ and $f(\varrho^2) \cos 2\varphi$ and are obtained according to the same procedure. Amplifier levers permit the variation of the relative importance of these five terms. The displacement of the tape presents the path difference $\triangle$, and if the ribbon is wound on a pulley of suitable radius, the rotation of which is equal to the difference of phase ψ, it is easy to perform the integration of the quantities

Accordingly, this machine makes it possible to determine the distribution of the light in a small area surrounding the paraxial focus in the presence of different geometric aberrations, isolated or superimposed. To do this, the computer would install on the apparatus cams representing the results of his calculations. Next the diffraction factor would be determined systematically, each point requiring an operation taking 8 to 10 minutes depending upon the individual case. We have checked the operation of the machine by employing calculations already done by different authors (Airy's work on diffraction; and Nijboer's work on curvature of field, spherical aberration, coma and astigmatism). The absolute error in the amplitude of vibration generally does not exceed 5×10^{-3} which corresponds to an

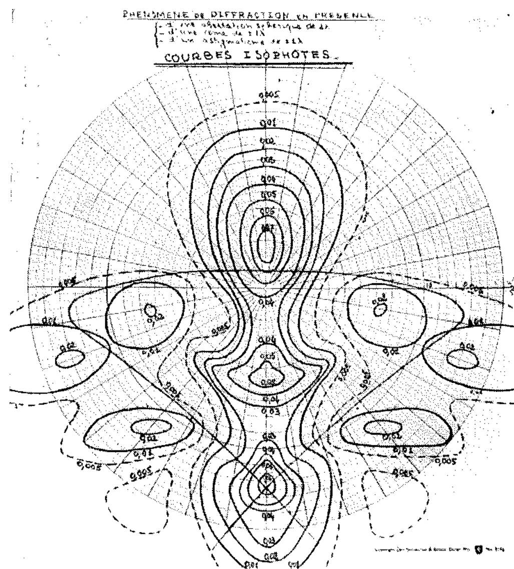

Fig. 2.

error of intensity usually less than the following values:

$\Delta I < 0{,}01$ $\Delta I < 0{,}003$ $\Delta I < 0{,}001$
if $I \doteqdot 1$ f $I \doteqdot 0{,}1$ f $I \doteqdot 0{,}01$, *etc.*

The precision of the apparatus is accordingly entirely sufficient for practical purposes.

The apparatus has been used to determine the structure of diffraction patterns in the following cases:

1°. In presence of various third order aberrations: cases of spherical aberration, coma, astigmatism have been studied and the diffraction pattern was in every case compared with the geometrical diffusion. It is found that a small astigmatism leads to a diffraction pattern being closely related to the focal line of geometrical optics. In the case of coma, and moreover in the case of spherical aberration, it is necessary that the optical path differences should be relatively important (5 to 10 wavelengths) in order that the pattern would be nearly the geometrical one.

An example of superposition of various aberrations is given in Fig. 2 (2 λ of spherical aberrations, $\pm$ 1 λ of coma, $\pm$ 1 λ of astigmatism).

2°. To determine the best way of "correcting" spherical aberation in optical systems. A similar study of sine condition is to be performed very soon.

3°. Practical cases corresponding to optical systems being designed: Among them, microscope objectives for the Bausch and Lomb firm or Schmidt cameras for the Cambridge Observatory are to be noticed.

Discussion.

A great number of questions in close connection with the above paper, concerning the resolving power in optical and electronic instruments. Prof. Gabor gave excellent following summary of the discussion.

EMISSION MICROSCOPY IN METALLURGICAL RESEARCH.

W. G. BURGERS, Laboratory for Physical Chemistry, Delft, and
G. W. RATHENAU, Philips Research Laboratories, Eindhoven.

As early as in 1935 the phase transformation of iron at 900° C was studied in the Philips' laboratories by cinematographical recording of the image given by an electron emission magnifier. In addition phase transformations in other metals as well as the dynamics of recrystallization were studied by electron optical means. The results of these investigations were published in several papers: [1—9].

Fig. 1 shows two stages in the phase transformation of γ into α -iron as observed with an electron emission magnifier in 1936.

It has since been the aim of several workers to increase the resolving power and magnification of the emission microscope above the limits set to light optical instruments.

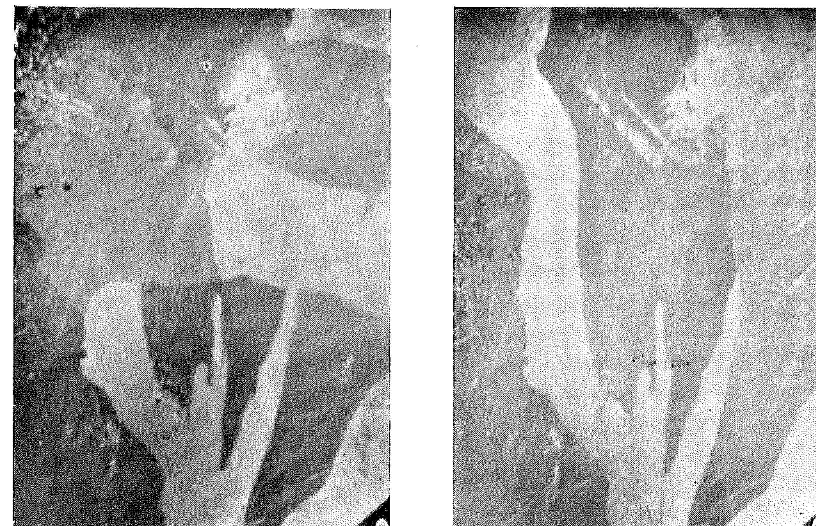

Fig. 1. Two stages in the phase transformation of γ into α iron as observed with an electron emission magnifier (Burgers and Ploos van Amstel [9]). The boundary which divides the growing α and the disappearing γ phase, seen in A. has on cooling (B) moved up.

It has been pointed out especially by Recknagel [10, 11] that the emission microscope is liable to specific aber-

rations. Because of the strong accelerating field even those electrons which leave the cathode at right angles to the axis, may take part in image formation. Since on the other hand the velocity of thermal electrons is not negligible near the cathode surface the electron rays, coming from one object point, do not correspond to one virtual image point behind the cathode surface. Geometrical optics show, that the spherical aberration of the accelerating field makes the object point to appear as a circle of approximate diameter ε / E, where ε is the most probable thermal energy of the emitted electrons in electronvolts and E the electric field near the cathode surface. For thermal electrons with $\varepsilon = 0.1$ and a field strength of 100 kV/cm, which is at present realizable, this leads to a resolution of 10^{-6} cm. However, the wave character of the electrons must also be taken into consideration. It has been shown by Recknagel that at high field strengths the dependence of the diameter of the circle of confusion on the field strength is much less than indicated by the above formula and that the resolution will not surpass the value set by the wave length of the emitted electrons near the cathode surface.

In metallurgical research Mecklenburg, Boersch and Kinder have obtained resolutions of a few hundred A.U. by using high field strength.

At the Philips' laboratories an emission microscope is being developed, which gives about the same resolution. This instrument has an accelerating field and three magnetic lenses.

Several investigations are in progress. In a preliminary investigation the $\gamma - \alpha$ transition of iron, which was formerly studied by Burgers and Ploos van Amstel, has again been examined. Instead of a steep temperature gradient such that α—grains were always present in the colder parts of the specimen, a smaller temperature gradient has now been used. In such a way not only the growth but also the nucleation of α-crystals could be followed.

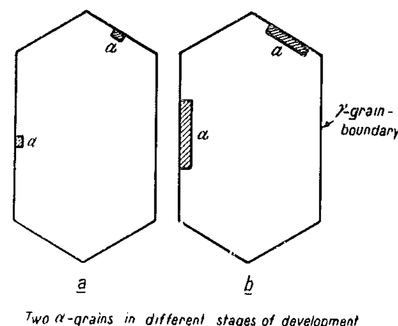

Fig. 2. Formation and initial growth of nuclei of the α phase of iron, schematically.

Fig. 2 shows schematically that probably α-nuclei form on, and preferently grow along, the γ-grain boundaries. Thus the disturbance of the atomic arrangement at the grain boundaries may facilitate not only the formation but also the growth of grains of a new phase.

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Discussion.

Prof. J. A. Prins: In Burgers' film on Ni-Fe alloys the direction of growth lies in the twinning plane of the newly formed twins, while in Rathenau's experiments on Fe it lies along the old grain boundaries. These results are conflicting.

Answer: The experiments taken are of a different kind. In the film on recrystallization of Ni-Fe alloys, germs of face-centered material, which were probably present before heating, grow in a face-centered matrix of grains with a sharp texture and therefore with low surface energy. In the phase transformation of iron, nuclei of a new phase are formed on the grain boundaries which, because of the absence of texture, have high surface energies and allow of quick growth along their surface.

Prof. Palacios: Would it be possible to excite the electronic emission of metals by means of the photoelectric effect?

Answer: This is certainly possible and has been done originally by J. Pohl. As shown in the formula given the initial velocity of the emitted electrons must be kept small to avoid too large a circle of confusion.

Prof. Grivet: How is the surface rendered emitting at comparatively low temperatures and in a continuously evacuated apparatus?

Answer: Evaporation of Cs allows emission at about 500° C.

CORRECTION AND DETECTION OF ASTIGMATISM.

J. B. LE POOLE and W. A. LE RÜTTE, T.P.D., E.M.Div., Delft.

A high resolving power requires a lens which is sufficiently free from astigmatism. For 10 A.U. resolution the distance between the focal lines must be less than about 0.25 micron. It is hardly possible to obtain this by careful machining.

As indicated by many others, it is therefore necessary to correct the lens for astigmatism. This may be done mechanically as Hillier did, which is inconvenient for electrostatic lenses, or by introducing an auxiliary field either magnetic or electrostatic.

Most suitable is a transverse field, with two perpendicular planes of symmetry, as this field acts as a lens having no power but only astigmatism. The field can be derived from two pairs of electrodes or pole pieces, as indicated in Fig. 1.

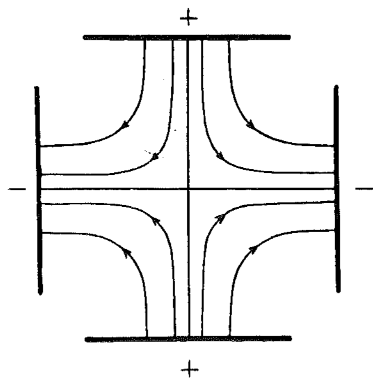

Fig. 1.

Generally spoken the direction of the focal lines of this field will not correspond to that of the astigmatism of the lens. Changing the direction would be feasible by turning the electrodes or pole pieces of the auxiliary field.

It is easier, however, to use a second and similar set and to change the ratio of fieldstrengths. It is obvious, that the two sets should not be put at 90 degrees, as they would counteract each other.

It seems logical to put them at 45° degrees so as to have a symmetrical arrangement.

The set-up is very easy in operation as was shown by a demonstration. It proved convenient to have one knob for changing the direction and another for changing the amount of astigmatism.

A second problem is to detect astigmatism.

As the aperture of the illuminating beam is very small, astigmatism is hardly visible on the screen. It does show, however, on micrographs due to diffraction.

The idea of our method is to increase the illuminating aperture, thereby increasing the geometrical astigmatism. This is done by a further development of the "wobbler" device, where, by a double set of deflectors, the illuminating beam was

given a rapidly changing tilt. In this focussing system the tilt changed only in one plane. Addition of a second set perpendicular to the first and energised with 90 degrees phase difference, makes the illuminating beam rotate in a cone. As the half angle of this cone can be much larger than the aperture of the beam entering the objective lens without "wobbler" the astigmatism increases considerably.

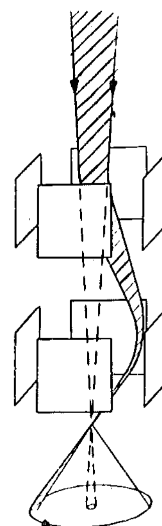

Fig. 2.

If an image is formed of an object with small holes, *e.g.* a diatom, and the objective current is changed, the image of the hole will describe a circle with varying diameter if the lens is free of astigmatism.

If there is astigmatism, however, the well known ellipses appear on the screen. The two focal lines show up at two particular objective currents. From this it is possible to determine the distance between the focal lines.

In the Delft microscope the focal lines can be made to coincide by correcting screws outside the vacuum.

Experiments showed that this way of correction is very quick. As astigmatism changes with time (contamination) and also with the specimen (charging) the question arises whether it pays to correct astigmatism every time a particularly good picture is wanted.

Comparison with the fringe method for detecting astigmatism shows an advantage of the "wobbler" method as long as the astigmatism is not too small.

This follows from simple calculations. The width of the first fringe is $d = \sqrt{\lambda a}$ where a is the distance between the focal lines. Suppose $a = 1\ \mu$ and $\lambda = 0.06$ A.U. (100 kV). Then d = 20 A.U. ≈ resolving power of the microscope.

Now let us compare this to the wobbler device where the half angle of the cone is $\alpha = 1 : 200$. Focussing on one focal line will then make the other visible with a length $d_1 = 2\alpha a = 100$ A.U. Moreover the angle α could be increased to obtain still better accuracy.

Discussion.

Prof. Grivet remarks that the use of fields has been investigated and published in France a.o. by Bertein.

Answer: The aim of this paper was to show how easy in operation the correction method is.

Prof. Gabor: Is there any reason to limit the swing to an angle of 1 : 200? Could the method not be made more sensitive than the Hillier-Ramberg fringe method?

Answer: Limitation is necessary if a physical objective aperture is used.

THE USE OF MARGINAL RAYS FOR THE STUDY OF ASYMMETRY IN ELECTROSTATIC LENSES.

P. GRIVET, F. BERTEIN and E. REGENSTREIF, Paris.

We observed in 1947 a new optical phenomenon which appeared in particular on a plate offered at the British Meeting of Electronic Microscopy in September 1948 [1]. We will describe it below and give an elementary theory.

Let us consider a pencil of rays coming from a point of the axis Oz, passing through an electrostatic lens in the electron microscope. In the case in which the voltage V of the central electrode L is close to that of the source cathode, or more negative, the trajectories of the rays are shown on Fig. 1; as one gets further away

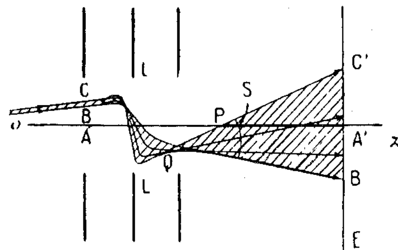

Fig. 1.

from the axis Oz the "usual" rays between AA' and BB' are observed; those rays give the images on the screen E (the extreme off axis rays which give the corresponding aberration must be eliminated). Between BB' and CC' one observes the rays whose impact points on E have an inverse displacement with respect to the normal case when going from B to C; their path in the lens is more sinuous. The theory [2] indicates that more off axis incident rays should have more oscillations. Actually, the latter exist only in such a narrow zone that they cannot be observed, but it is possible to show experimentally the existence of rays between BB' and CC' (shaded zone on the figure); they are responsible for the patterns shown (after suitable magnification) of which we are going to give an elementary proof.

Theory of the phenomenon

The two parts of the caustic corresponding to BB'CC' are:

1. the A'P part of the axis
2. an axially symmetric surface, the section of which is B'Q.

Where those two parts cross the screen E there is an accumulation of "light" superposed on the optical effects (images) due to the usual rays AA'BB'; the corresponding wave surface S is axially symmetric.

Actually, the considered rays pass close enough to L with a reduced speed in such a way that it should be expected that they would be very sensitive to the action of the field accidental asymmetries, those asymmetries are related to the deviation

of L from the axial symmetry. It is known that those asymmetries give a perturbing potential $\varphi\,(rz\Theta)$ superposed on the normal potential; φ is the sum of terms as $\cos\,(\Theta-\Theta_1)$, $\cos 2\,(\Theta-\Theta_2)$ ... $\cos m\,(\Theta-\Theta_m)$ ... as one turns around Oz.

We shall assume first that $\varphi\,(rz\,\Theta)$ contains only one m order term. Therefore it has an m order repetitive symmetry on the wave surface S; it can then be shown that the equation of the useful sector of S has the form, in cylindrical coordinates):

$$z = F(r) + a_m \cos m\,(\Theta-\Theta_m')$$

then a simple geometrical study gives the luminous pattern in the E plane due to the rays. Let us consider a set of rays leaning on the section of S the azimuthal angle of which is Θ. In the E plane this gives a straight line D with the same azimuth Θ and this line does not cross Oz. The distance between D and Oz takes the form $b_m \sin m\,(\Theta-\Theta_m')$. When the section of S takes all values of Θ, D turns of a value 2π, the enveloppe H of D is an hypocycloïd centered on Oz; H is now the section in E of the central nappe of the caustic shown by the accumulation of light; the section of which is A' in the case of axial symmetry (the outer nappe of the caustic is outside of the plate).

m = 1, this defect can be given by electrode misalignment, H reduces to a point A'' as in the revolution case: D turns around a point different from A': the general luminous pattern in E is not perturbed but for the lateral displacement.

m = 2; this defect may arise from the ellipticity of the holes. H has 4 cusps. One would expect this case to be easily observed, due to the great importance of the defects of order 2 (ellipticity) in lenses.

With m = 3, there are 3 cusps.

In the general case the closer the rays to L the greater number of m order effects are superposed; H is a more or less complicate curve with cusps [3].

Let V be varied in such a way that the rays BB'CC' be at first very close to the axis Oz, then progressively approach L. At first, only the order 2 effects are observed. Then order 3 effects appear and are progressively superposed on order 2. The distance between D and Oz is then of the form:

$$b_2 \sin 2\,(\Theta-\Theta_2') + b_3 \sin 3\,(\Theta-\Theta_3')$$

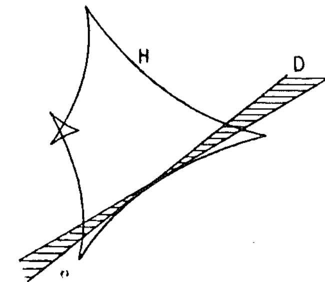

Fig. 2.

Fig. 2 shows the phenomenon; H is a rather simple curve. Finally high order m effects appear, and H becomes complicated.

In the case of the plates shown the pattern is symmetrical, which suggests the existence of a common plane of symmetry for order 2 and 3 components. This is not an accidental occurrence, as the perturbating field in this case, was due to the high voltage lead to the lens, which had purposely been made of exaggerated size. This power supply lead obviously imposes its plane of symmetry [4].

In the case where shape defects of the electrodes appear on H, it is of

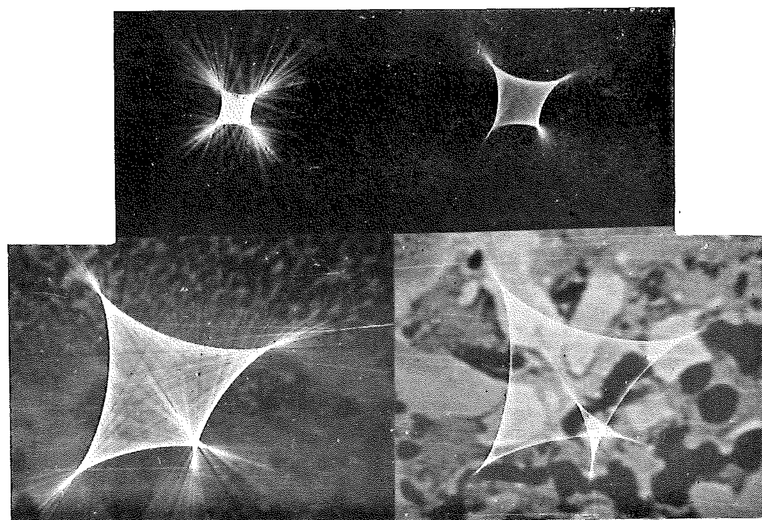

Fig. 3.

course to be expected that no symmetry will appear for the different order components are in this case of random mutual orientation.

The more or less thin black beam patterns are due to the fact that the traces D are absent in some intervals; this is because some groups of rays in the BB'CC' zone are missing due to diaphragms.

Application: The measurement of astigmatism due to ellipticity

It is of course to be expected that the examination of H versus V may be a very sensitive way to study the asymmetry defects of lenses. This study is of great interest when one is looking for the best resolving power.

The theory can be carried further by replacing the lens by a hyperbolic model.

It is possible to evaluate approximately the phenomenon, when it is due to the ellipticity of the central electrode. Then:

$$R/L \approx \eta$$

when R, radius of the H curve

L, distance between lens and screen

η, ellipticity of the electrode hole.

In general the H curve is easily measurable if necessary by using further magnification by means of another lens.

References.

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2. Bush and Bruche, Beiträge zur Electronenoptik, Leipzig 1937.
3. F. Bertein and E. Regenstreif, Compt. Rend., 228 13th June 1949) 1854.
4. F. Bertein, Compt. Rend. 229 (25th July 1949) 291.

Discussion.

Ir Le Poole: How can the effect of higher order errors be avoided?

Answer: According to the next, the marginal "oscillating" rays BB'CC' must run "far" from L, so as to undegro no dissymmetry but the elliptical one m = 2; that requires to give a sufficient value to the negative voltage of L.

THE ABERRATIONS OF MAGNETIC ELECTRON LENSES DUE TO ASYMMETRIES.

by P. A. STURROCK, Cavendish Lab., Cambridge, England.

Abstract: The mathematical problem of examining the aberrations of a magnetic lens caused by arbitrary small asymmetries is dissected and the treatment of each constituent briefly indicated. As an example, the tolerance on ellipticity is obtained for a typical objective lens.

1. Defects in the machining of magnetic lenses result in aberrations which may usurp the rôle of spherical aberration in limiting resolution. This paper outlines the mathematical examination of this effect; it is based on Bertein's work for electrostatic lenses[1]. The working model comprises only the lips of the pole-pieces, which are assumed to be surfaces of constant scalar potential.

2. The perturbation of a field due to perturbation of two of its equi-(scalar)-potential surfaces may be found as follows:

Let the spatial function $\varphi(x)$ satisfy the Laplace equation

$$\nabla^2 \varphi(x) = 0, \tag{2,1}$$

and let P be the general point of two equipotential surfaces α and β. Upon perturbation of α and β, characterised by a parameter λ, P may be considered to move along the outward normal to P^*, where

$$PP^* = \lambda\, n^I + \tfrac{1}{2}\lambda^2\, n^{II} + \dots \tag{2.2}$$

If the perturbation changes $\varphi(x)$ to $\varphi^*(x)$, we may similarly write

$$\varphi^* - \varphi = \lambda\, \varphi^I + \tfrac{1}{2}\lambda^2\, \varphi^{II} + \dots \tag{2.3}$$

Now φ^* (P^*) is expressible as a Taylor series in PP^* with coefficients $\varphi^*(P)$, $\varphi^*_n(P)$, $\varphi^*_{nn}(P)$, etc., where the suffix n denotes differentiation along the normal. Noting that $\varphi^*(P^*) = \varphi(P)$, and using (2.2) and (2.3), one finds that

$$\varphi^I(P) = -\, n^I(P)\, \varphi_n(P), \tag{2.4}$$

$$\varphi^{II}(P) = -\, n^{II}(P)\, \varphi_n(P) - 2\, n^I(P)\, \varphi^I_n(P) - \{n^I(P)\}^2\, \varphi_{nn}(P), \text{ etc.}$$

Hence we may find the coefficients $\varphi^I(x)$, $\varphi^{II}(x)$, etc. of the series (2.3) by solving the Laplace equation (2.1) with boundary conditions given by equations (2.4). It proves useful to note that the ratio of $\varphi_{nn}(P)$ to $\varphi_n(P)$ is the first-curvature of the equipotential surface at P.

3. Let the z-axis of cartesian axes x, y, z, approximate to an axis of rotational symmetry of the lens. We may combine x and y into a complex number u, or change to cylindrical coordinates, by

$$(3.1) \qquad \bar{u} = x - iy, \quad u = x + iy; \quad \text{or} \quad x + iy = re^{i\Theta}$$

In finding the field of given pole-pieces, the Fourier expansion

$$(3.2) \qquad \varphi = \sum_{k=0}^{\infty} \{\bar{\psi}_k e^{ik\Theta} + \psi_k e^{-ik\Theta}\}$$

is most useful. Since φ satisfies the Laplace equation (2.1), one finds that the $\psi_k(z,r)$ must satisfy the cylindrical equations

$$(3.3) \qquad \left\{\frac{\partial^2}{\partial z^2} + \frac{\partial^2}{\partial r^2} + \frac{1}{r}\frac{\partial}{\partial r} - \frac{k^2}{r^2}\right\} \psi_k = 0, \qquad k = 0, 1, 2, \ldots\ldots$$

To examine the electron-optical properties of a magnetic field, one needs the vector potential. The only field equation this satifies is

$$(3.4) \qquad \text{curl curl } \mathbf{A} \equiv \{\text{grad div} - \nabla^2\} \mathbf{A} = 0,$$

but it is customary to assign also the Lorentz condition

$$(3.5) \qquad \text{div } \mathbf{A} \equiv \frac{\partial}{\partial \bar{u}}(A_x - iA_y) + \frac{\partial}{\partial u}(A_x + iA_y) + \frac{\partial Az}{\partial z} = 0.$$

The relation (3.5) is satisfied if and only if $\mathbf{A}$ is derivable from a „complex potential" U according to the equations

$$(3.6) \qquad A_x + iA_y = -\frac{\partial U}{\partial u} \text{ and } A_z = \frac{\partial \bar{U}}{\partial \bar{u}} + \frac{\partial U}{\partial u}.$$

We see from (3.4) that U must satisfy the Laplace equation which, in complex coordinates, has the form

$$(3.7) \qquad \nabla^2 U \equiv \left\{\frac{\partial^2}{\partial z^2} + 4\frac{\partial^2}{\partial \bar{u}\, \partial u}\right\} U = 0.$$

Since also the scalar potential is derivable from U according to

$$(3.8) \qquad \varphi = -i\left\{\frac{\partial \bar{U}}{\partial \bar{u}} - \frac{\partial U}{\partial u}\right\},$$

we find that the general expansion for U is of the form

$$(3.9) \qquad U = \sum_{h \geq e \geq 0}^{\infty} \frac{i(-)^{l+1}}{2^{h+1}(h+1)!\, l!} \bar{u}^{h+1} u^l \Phi_{h-l,\, 2l}$$

wherein the $\Phi_{k,j}(z)$ are the j'th derivatives of the complex fuctions $\Phi_k(z)$ which are related to the $\psi_x(z,r)$ by the limiting relation.

$$(3.9) \qquad \Phi_k(z) = 2^k \cdot k! \lim_{r \to 0} \{r^{-k} \psi_k(z, r)\}, \qquad k = 0, 1, 2, \ldots$$

The coefficient $\Phi_0(z)$ may always be chosen to be real.

4. Whereas the single coefficient ψ_0 suffices to represent the field of a symmetrical lens, asymmetries require the introduction of terms ψ_1, ψ_2, etc., which may be assossiated with the φ^{I}, φ^{II}, etc., previously introduced. Hence the field of an asymmetrical lens is found by consecutive solution of the cylindrical equations (3.3) for which the boundary conditions are obtained from equations (2.4).

In order so to use equations (2.4), one must assign Fourier coefficients also to the asymmetrical pole-piece surfaces. Thus if the cylindrical faces suffer a distortion represented by

$$(4.1) \qquad r - a = \varepsilon r^{I} + \tfrac{1}{2}\varepsilon^2 r^{II} + \ldots,$$

one must expand the coefficients $r^{I}(z, \Theta)$, etc., in the form

$$(4.2) \qquad r^{I} = \sum_{k=0}^{\infty} \{\bar{R}_k^{I} e^{ik\Theta} + R_k^{I} e^{-ik\Theta}\}.$$

The boundary conditions for the $\psi_k(z,r)$ then involve the $R_k^{I}(z)$, etc.

If the coefficients R_k^{I}, R_k^{II}, etc., $k = 1, 2, \ldots\ldots$, are all comparable, the most important aberration is the astigmatism which is due to R_2^{I}, the first *ellipticity* coefficient. Hence in examining distortion, it suffices to consider only ellipticity and to work only to the first order.

If, however, the lens suffers from eccentricity, there is no R_2^{I} term and it is, in fact, necessary to work to the second order.

5. Electron rays in static fields are derivable from

$$(5.1) \qquad \delta \int_0^B \{\varrho - \mathbf{A} \cdot s_1\}\, ds = 0$$

wherein ϱ is the H—ϱ momentum of the beam; $\mathbf{A}$ is the vector potential; s is arc-length; s_1 is the unit tangential vector; and δ refers to small changes of the path which leave the end-points invariant. Since ϱ is constant, we may measure $\mathbf{A}$ and ϱ in units of ϱ, so that ϱ itself becomes unity

By using (3..6) and (3.9), we may rewrite (5.1) as

$$(5.2) \qquad \delta \int_0^B F\, dz = 0$$

wherein F is a power series in $\bar{u}$, u, $\bar{u}'$, and u', where a prime denotes $\frac{d}{dz}$.

We may separate F into F_g and F_β of which F_g comprises all terms up to the second order except the elliptic terms $\bar{u}^2$, etc., and F_β comprises all other terms. Then F_g, the „Gaussian function", is responsible for image formation whereas F_β, the „non-Gaussian function", is in general responsible for aberrations.

Investigation of F_g enables us to introduce a „canonical" complex coordinate w, linearly related to u, such that the Gaussian rays are the solutions of the differential equation

$$(5.3) \qquad \left\{\frac{d^2}{dz^2} + \Phi^2_{0,1}\right\} w = 0.$$

If we regard F_β as a perturbation of F_g, we may evaluate the aberrations, to the first order in their magnitude, by means of a „perturbation eikonal", Y, introduced as follows:

Let n be the conjugate variable to w defined by

$$n = \frac{\partial Y}{\partial \overline{w}'} \quad \text{wherein } L \equiv L(\overline{w}, w, \overline{w}', w', z) \equiv F(\overline{u}, u, \overline{u}', u', z). \tag{5.4}$$

Then n, the „complex ray coordinate", instead of w, will characterise the properties of the lens when it is focussed at infinity. The aberration component, ν, evaluated at any plane Z_b of the image space, is derivable, to the first order in its magnitude, from

$$\nu_b = \frac{\partial Y}{\partial \overline{w}_b}, \tag{5.5}$$

wherein Y is the „point perturbation eikonal" defined by

$$Y \equiv Y(\overline{w}_o, w_o, \overline{w}_b, w_b, z_o, z_b) = \int_{z_0}^{z_b} L_\beta \, dz, \tag{5.6}$$

where the integral is evaluated along the Gaussian rays.

6. Only the wholly aperture-dependent aberrations are important in an objective lens. To the first order in its magnitude, the aperture-dependent part of the eikonal Y proves to be expressible in the form

$$Y = \begin{pmatrix} 1 \\ \overline{w}_b \\ \overline{w}_b^2 \end{pmatrix}' \begin{pmatrix} K & \overline{\mathbf{R}} & \overline{\mathbf{Q}} \\ \mathbf{R} & M & \overline{\mathbf{P}} \\ \mathbf{Q} & \mathbf{P} & N \end{pmatrix} \begin{pmatrix} 1 \\ w_b \\ w_b^2 \end{pmatrix}, \tag{6.1}$$

in which, since Y is essentially real, K, M, and N are real, but **P**, **Q** and **R** are complex. Using (5.5), the aberrations due to (6.1) are

$$\nu_b = \begin{pmatrix} 1 \\ 2\overline{w}_b \end{pmatrix}' \begin{pmatrix} \mathbf{R} & M & \overline{\mathbf{P}} \\ \mathbf{Q} & \mathbf{P} & N \end{pmatrix} \begin{pmatrix} 1 \\ w_b \\ w_b^2 \end{pmatrix}. \tag{6.2}$$

Inspection of (6.2) shows that M represents defocussing: N is third-order spherical aberration; **P** is coma; **Q** is astigmatism; and **R** is purely a displacement of the image.

Bij sorting out the terms of the integral (5.6), one finds that

$$N = -\tfrac{1}{4} \int_{z_0}^{z_b} \{H^2 - h^4 \Phi_{0,1} \Phi_{0,3}\} \, dz \qquad \text{wherein } H = h'^2 + h^2 \Phi_{0,1}^2, \tag{6.3}$$

$h(z)$ being the solution of (5.3) which is zero at z_o and unity at z_b. (Differentiation of H shows it may be easily computed by indefinite integration).

If the lens suffers only from ellipticity, **P** and **R** must vanish, and the integral for **Q** becomes simply

$$\mathbf{Q} = \tfrac{1}{4} \int_{z_0}^{z_b} h^2 \Phi_2 \, e^{2i\Phi_0} \, dz. \tag{6.4}$$

7. Computations have been performed for pole-pieces whose inner corners meet the z—r plane in the points ($\pm$ a, $\pm$ a), i.e. whose spacing is equal to its bore-diameter. In examining the lens as an immersion objective lens, the object was at (— a, 0) and the lens focussed at (+ ∞, 0). The focal length, f, and N were then given by

$$f = 1.33 \text{ a and } N = -0.20 \text{ a}^{-3}. \tag{7.1}$$

Also, if the lens suffers from ellipticity of amplitude Δ,

$$\mathbf{Q} = 1.45 \, \Delta \, a^{-2}. \tag{7.2}$$

If α is the semi-aperture-angle, the diameter of disc of least confusion and length of the astigmatic lines, referred back to the object plane, are given respectively by

$$d_o \equiv |N| \, f^4 \alpha^3 = 0.65 \, a \, \alpha^3 \quad \text{and} \quad l_o \equiv 8 \, |\mathbf{Q}| \, f^2 \alpha = \Delta \, \alpha, \tag{7.3}$$

Hence if a = 0.25 cms (i.e. the bore 5 mm) one must make $\alpha = 0.01$ in order that $d_o = 20$ Å. Then to make $l_o = 20$ Å also, Δ must be 0.01 microns. Thus, for the astigmatism due to ellipticity to be less important than the spherical aberration, the overall variation in the bore of the pole-pieces must be less than 0.02 microns.

The author is indebted to the Associated Electrical Industries, Alder maston, and to the Department of Scientific and Industrieal Research for the means to carry out the above investigation.

References.

1. F. Bertein, Ann. de Radioel., 2 (1947) 379.

Discussion:

In discussion, Ir J. B. le Poole pointed out that the tolerance mentioned by Mr Sturrock seemed to be more stringent, by a factor of about 10, than that given by Glaser. Mr Sturrock replied that the regions of the polepieces much the most sensitive to distortion are the neighbourhoods of the sharp corners. Rounding of these corners may very well improve the tolerance on ellipticity. This, he thought, explained the discrepancy mentioned since the model used by Glaser was in fact rounded.

in Ladd and Braendle's, 103 m/sec, and in Fullam and Gessler's 336 m/sec. The velocity of sound generally is well above these values (*e.g.* in cork, 430 m/sec, in beeswax, 880 m/sec, in cotton cord, 1425 m/sec) but in caoutchouc at 30—60 m/sec it is exceptionally low and some of the more successful pictures published of cut sections have been of rubbers.

Section cutting is a general method for obtaining thin layers of material but in special cases there are easier ways of obtaining thin lamellae. Skin and cuticle tissues have a laminated structure and can be mechanically stripped into layers or separated chemically by acid or enzyme treatment; cells grown in tissue culture develop in a thin enough layer for direct electron microscope examination. Sometimes a suitable film formed on a specimen surface may be peeled off, splitting away a layer of the material with it. This has been done in the case of bacteria with a beryllium film and further serial sections of the same organisms can be obtained by repeating the process. A surface layer of vulcanised rubber can be torn up in a pellicle of gelatine which is then dissolved in water after applying a strengthening film of formvar to the rubber.

These latter methods come very close in practice to the replica methods by which surface structures of a massive object are studied as an impression on a thin plastic or other film. Modifications are now available suitable not only for hard dry materials such as etched metals but also for soft tissues and recently even for wet tissues. By making replicas of cut or stripped surfaces of tissues structures underlying the original surface can be studied so that the method is an extremely powerful one.

Where other methods fail the material may be broken up by more random methods such as milling or the use of supersonic vibrations. It will then tend to split along lines of natural weakness.

In many industrial applications the specimen is already in a sufficiently finely divided state and materials such as pigments and carbon blacks only require dispersing and displaying on the film. In other cases the specimen may be produced *in situ* either as a thin film or as a sublimate or smoke and these are probably the easiest of all specimens to handle.

The electron beam cannot be operated in air because of its low penetration of matter and the specimen must withstand vacuum conditions. We cannot therefore observe living activity such as the method of propulsion of a motile bacterium, which is in contrast to optical microscopy where the specimen may be immersed in liquid if desired.

Set out in Table I is our analysis of the consequences of the low beam penetration for the preparation and mounting of specimens.

The second consideration in comparing electron and optical microscope specimens is the difference in the method of image formation and development of contrast. In optical microscopy the main effects are due to variations in refractive index and absorption (including colour); in electron microscopy contrast is mainly due to electron scattering, and the two instruments will not necessarily show the structure of a specimen in the same contrast relationships. Spores and granular bodies in bacteria for instance appear light under the optical microscope but densely black in the electron microscope.

TABLE I

The Electron Beam has low penetration:
Therefore:
- The Specimen is *mounted on a thin film* which requires a *supporting grid.*
  - This involves a *small and discontinuous area for search* and, a need for *micro-location* of some specimens.
  - *Manipulation* may *not* be *directly on* the thin *mount* and, means of *transfer to the final mount* are needed.
- *The Specimen itself must be thinner* than for Optical Microscopy.
  - This involves *redevelopments in Microtomy* and, development of *other means of thinning* or *comminuting* material.
  - Use of *Replica Methods* for surfaces.
- The *Microscope must be evacuated* so that,
  - the *Specimen must be dried,*
  - *Active Life is not observable* and,
  - *Volatile or Sublimable Specimens are unsuitable.*

The variations in electron scattering power in a specimen depend upon the thickness, the mass density and the nature of the atoms present (heavy atoms being more efficient electron scatterers than light ones). Since most organic materials consist mainly of carbon, oxygen and nitrogen all of which have similar scattering powers variations of contrast in a picture of organic material are due almost exclusively to variations in thickness and density. A suitable reagent containing heavy atoms may however, react with groups at particular regions in the specimen which thus becomes "stained" to show these regions. Thus Phosphotungstic acid has been used to show periodicities in muscle fibres. Different reagents may be absorbed preferentially at different points but as the effects of the different stains are indistinguishable there is no analogue in electron microscopy of the method of counterstaining in contrasting colours used in optical work.

This increase in contrast conferred by heavy elements is an important factor in the popularity of the method of shadowcasting. The resolution of two points is most efficient when the contrast of each with the background is high and the increased definition of surface detail due to the presence of the shadowing metal is as valuable a feature as the revelation of the heights and shapes of protuberances in the forms of the shadows.

Discussion.

Dr Thewlis drew attention to the high toxicity of inhaled Beryllium vapour and dust.

Answer: This warning against Beryllium poisoning and its possible delayed effects is most important.

Dr Brown advocated the use of thermally stable Al_2O_3 films to support specimens which may overheat in locally thick regions and cause breakage if a plastic film is used, so prejudicing examination of the thinner regions.

Answer: Al_2O_3 films are one solution of this difficulty. Another is the use of Smiles'

"micro-isolation" method to remove the unwanted thick parts.

Mr Bengtsson. Supporting films of nitro-of triacetyl cellulose have a negative surface charge similar to that on cellulosic particles which therefore do not settle readily on them, and become aggregated in suspension. A layer of oxidised beryllium on the surface reverses the charge so that cellulosic particles are soon adsorbed from suspension without aggregation and the supernatant liquor can be drawn off.

Answer: I had not met this technique before. The principle seems capable of wider application.

Mr Sharpe asked whether a list could be compiled of liquids suitable for the dispersion of various powders.

Answer: I shall be glad to have information of this sort for possible incorporation in the book on techniques which I am preparing.

THE PREPARATION OF REPLICAS FROM METAL WIRES.

J. NUTTING, Cavendish Lab., Cambridge, Eng.

The dry stripping of plastic replicas from metal surfaces direct on to specimen mounting grids can be carried out satisfactorily only when the metal surface is larger than that of the grid. This difficulty has been overcome by a modification of the dry stripping technique described below.

The metal specimens used were steel wires which were mounted in "Perspex" before polishing a diametrical cross section. After etching and washing, 1 % Formvar in dioxane was flowed on to the prepared surface. The replica was cut at the "Perspex"-metal interface by scoring with a needle and then stripped from the metal with cellulose tape. The next stage was to remove the replica from the tape; this was carried out by dissolving

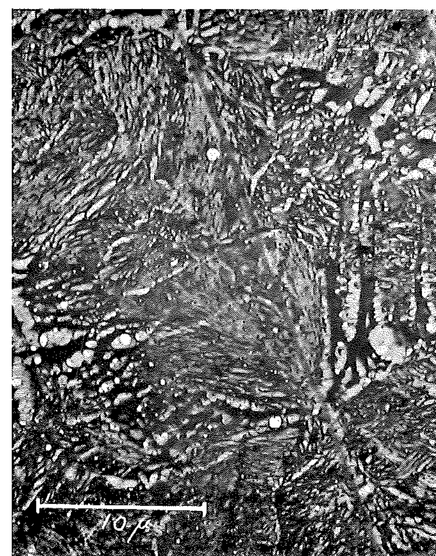

Fig. 1. 0.4 % steel wire in as patented condition. Longitudinal section. Etched 15 secs 4 % picral. Replica 1 % formvar in dioxane.

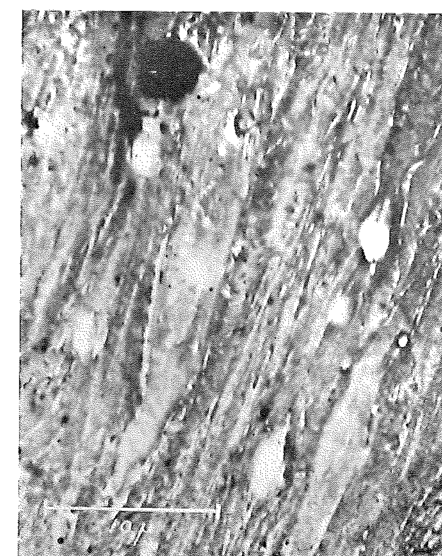

Fig. 2. Same as Fig. 1, after drawing down 75 %.

the glue from the tape with 60/80 petrol ether. As the glue softened it was possible to lift the replica from the tape with the aid of a needle. The replica was then transferred on a glass slide to another dish of petrol ether and allowed to wash for five minutes before mounting on the specimen grid.

This technique has been found suitable for removing replicas from steel wires 1—2 mm. in diameter. The results obtained with a 0.4 % carbon steel wire are shown in Figs. 1 and 2. The first electron micrograph is that of a Formvar replica taken from the steel wire in the as patented condition; here the cementite lamellae are randomly orientated. Fig. 2 shows the structure obtained after a 75 % reduction in cross sectional area of the wire produced by drawing; in this case the lamellae are orientated along the drawing direction.

Discussion.

Mr Agar: Is the method capable of rendering visible any edge detail in metals as the Schaeffer method gives film thickening at the edge of the replica.

Answer: Since tearing of the replica at the Perspex-metal interface always occurs, this replica technique is not suitable for examining edge detail.

NEW METHODS FOR BIOLOGICAL REPLICAS.

E. KELLENBERGER, Physical Institute, Geneva.

Biological material is very sensitive to influences of various kinds *e.g.*: concentrated salt solutions (which occur always when a drop of a suspension in an isotonic solution is dried), distilled water, high temperature, desiccation, fixing agents, action of the electrons. Since it is impossible to eliminate these influences all at once, comparison studies must be made. To this end different preparation methods must be employed.

The effect of electron bombardment and desiccation may be eliminated with replicas. In 1948 we published a positive replica technique [1] which gives excellent definition without application of any pressure or high temperature. This method has since been improved and some auxiliary methods for the preparation of suspended particles have been developed. Our latest improvement is its application to wet specimens.

Brief description of the basic process.

A 0.2—0.3 mm. plate of some plastic material as well as the surface to be studied are wetted with an appropriate solvent of the plastic and then quickly put together. A good wetting solvent reaches all points of the surface. The molecules of the plastic finally replace the solvent. The latter diffuses through the plastic plate. After some hours the plastic plate is mechanically stripped off and coated in the usual way with SiO_2 or SiO. The plastic material is then dissolved. We made for this purpose a small distillation apparatus which considerably simplifies this manipulation. [2]

The shadow casting can be done before or after depositing the silica film and before or after dissolving the plastic.

The following combinations have been employed: Celluloid-acetone, methylmetacrylate-chloroform, polystyrene-halogenated hydrocarbons. We prefer the celluloid-acetone combination. Aceton has proved one of the best solvents because of its good wetting properties. Moreover it is miscible with water and therefore especially suited for wet specimens. Cellulose-acetobutyrate is better than the nitrate, the latter being plasticised with camphor, a very volatile substance unfit for vacuum coating.

This method can be used for all sorts of replicas of inorganic materials without any preliminary treatment. In particular one can make replicas of the inner walls of holes in case a surface is studied.

In the same simple manner we make replicas of smears on glass

slides. The specimen is slightly fixed in a flame. The adherence thus obtained allows crystals and impurities, due to the suspension medium, to be washed out. Also it prevents sticking of the object to the plastic plate.

It is obvious that fixing by heat produces alterations. We also make replicas of non fixed smears: the object sticking to the plastic plate is removed by enzymatic digestion and slight rubbing with cotton under water. We found that papaïn is suitable for a great number of preparations (chromosomes, blood cells, bacteria). This method may be applied to all objects not suspended in liquids. Fig. 1 represents chromosomes from the salivary gland of *Drosophila melanogaster*.

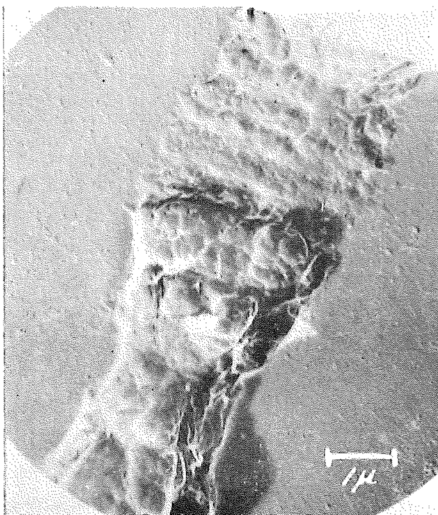

Fig. 1. Chromosomes from the salivary gland of *Drosophila melanogaster*, put on glass slide; digestion with papain to remove from plastic plate.

With suspensions we use the sediment of centrifugation. This is smeared on glass slides. If necessary these can be coated with albumin to procure good wetting and adhering. Excess water is carefully removed with blotting paper. (Replicas of droplets are sometimes obtained).

In another method we use a slide with a rather thick gelatin layer (*e.g.* fixed photographic plates). On top of this layer a drop of the suspension is put. The particles stay on the surface while the liquid diffuses in the gelatin. We then make a replica of the wet surface in the same manner as described above, using celluloid acetone. After several hours the plate is dried and the celluloid moulding can easily be stripped off. The objects have little tendency to stick to the celluloid. Fig. 2.

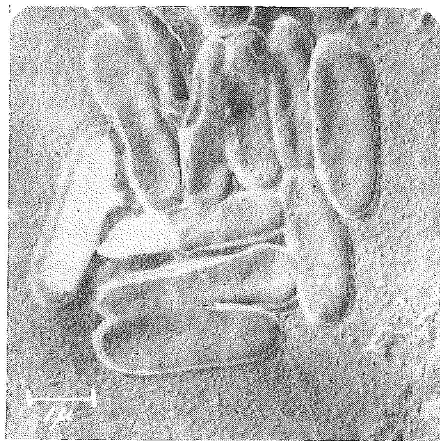

Fig. 2. Replica on gelatin of wet *Lactobacillus*.

By means of this method we found a.o. that bacteria shrink about 30 % in linear dimensions while drying. The use of acetone may be a source of artefacts indeed.

The application of this method is limited by the structure of the gelatin and the size of its pores.

A similar gelatin technique has been developed for the direct preparation of suspensions on the supporting collodion membrane. A gelatin layer (gelatin as used for photographic emulsions) is covered on one side by a 0.1 % solution of parlodion, collodion or formvar. After removal of the excess solution and evaporation the suspension is deposited as a drop on it. The preparation is kept in a moist atmosphere until all liquid has filtered through the thin film and has been absorbed by the gelatin. The preparation is then cut in little parts which are floated on water at about 50° C. The gelatin is dissolved and the pieces of collodion spread on the surface. The preparations are picked up in the usual way and dried on filter paper.

These methods have been developed under the direction of Prof. J. Weigle in the laboratory of electron microscopy of the University of Geneva. I thank the other members of the biological staff, especially V. Bonifas for many suggestions and the foundation Carl Hoffmann LaRoche for its material aid.

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MICROTOME SECTIONS OF ANIMAL TISSUES.

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This paper concerns a fairly simple method to make sections of 0.3 and 0,6 μ. These sections can be successfully examined even with an accelerating voltage of 50 kV. We based our investigation on the methods already long in use in histological research.

To make sections of this extreme thinness the following conditions must, broadly speaking, be fulfilled:

1. A microtome allowing sufficiently small shifts of the object must be available;
2. an embedding medium that facilitates the cutting of thin sections is needed;
3. the temperature of the object, the cutting blade, and the surrounding atmosphere must be kept down to about 10° C;
4. the object must be small, say about 1 mm.; and
5. it must be possible to mount these sections completely flat on the object holder of the electron microscope.

We used a 60 years-old microtome (Fig. 1), the so-called "Rocking Microtome" of the Cambridge Instrument Company, which we found in the Museum of our Institute. This microtome had no scale division into microns. Computing the minimum thickness of a section we came to a value of 0.6 μ. This thickness is obtained by shifting the mechanism by one cog of the cogwheel. It is also possible to adjust the automatic shift in such a way that the first section shifts by a cog and a half, that is 0.9 μ and the next one by half a cog, or 0.3 μ. In this way, therefore, we are able to cut sections of 0.3 and 0.6 μ. An exchange of views between the Cambridge Instrument Company and ourselves has resulted in that Company having undertaken to build a modern "rocking" microtome, following our suggestions for a number of improvements in the construction. With this instrument, which is based on simple principles, it will be possible to cut sections of 0.5 μ and even less.

To my mind the Rocking microtome, thanks to its special construction, is very suitable to serve as the prototype of an ultra-microtome because the movement of the cogwheel and the screw is transferred to the object on a reduced scale by means of a lever.

The second condition essential for cutting these thin sections is a suitable embedding medium. Pure paraffin, as generally used in histological work, is not suitable for our purpose since sections as thin as 0.6 μ are strongly compressed during cutting at room temperature, whereas at a lower temperature the paraffin

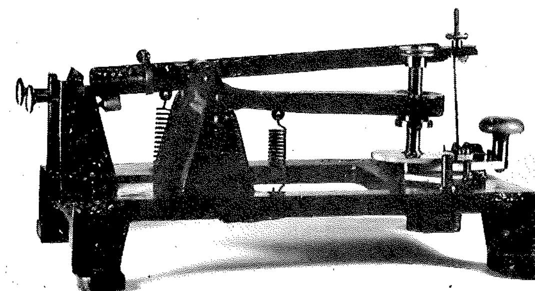

Fig. 1.

splinters. This drawback is overcome by using a mixture of 2 parts yellow beeswax and 1 part paraffin with a melting point of about 70° C. In this mixture the object is embedded in the thermostat.

Notwithstanding the higher melting point of this medium and the particular properties of beeswax, such as plasticity and viscosity, the optimal temperature for the actual cutting of the sections is less than 18° C. The most suitable temperature, in our experience, lies between 8° and 12° C. For this reason we cut the sections in unheated rooms during the winter. We have now, however, solved the problem of lowering the temperature by putting the microtome on an ice-cooled console, specially built for this purpose.

Since an electron microscope has a very small field of view, the object can be correspondingly small. Already before fixing the object we reduce it to 1—2 mm. So far we confined ourselves to the methods of fixation commonly used in histological research, as well as to the conventional methods of dehydration in the alcohol series and to the embedding in wax-paraffin already referred to.

Though not being able previously to cut sections of very hard objects such as bone we now succeed in doing so in the following manner. We first make 20 μ sections of the frozen or paraffin embedded object. Subsequently these sections are embedded in wax-paraffin in layers one above the other. We find that the resistance to the cutting blade is not greater now than that of softer objects. With this method of "two-step sectioning" we were able to

Fig. 2. Intestinal cell of *Ascaris* (a Roundworm).

make sections of 0.6 μ of the bone of large mammals.

So far we always used for cutting the ordinary plan-concave microtome blades which we ground on an oil-stone or hone.

The problem of mounting these sections has been greatly facilitated since fine copper gauze has been put on the market a.o. by Kodak.

Fig. 3. Nematocyst of *Corynactis* (a Sea Anemone).

These "grids" with a diameter of ⅛" are first provided in the usual manner with a parlodion membrane whereupon the section is mounted on it.

On being cut the sections are pinched together by the blade and more or less deformed. For this reason they have to be stretched and flattened before they are mounted. To this end we float the sections on distilled water at about 45° C. The softened sections are then stretched as a result of the surface tension. We then dip another collodion coated grid into the water underneath the section, catching the latter from below. After drying at about 50° C and dissolving the wax-paraffin in xylene, the sections are ready for examination in the electron microscope.

So far we have examined with the new technique the following tissues: intestinal epithelial cells of *Ascaris* and *Rana;* muscle of *Mytilus, Aeschna, Sagitta, Amphioxus* and *Triton;* gland cells (thyroid) of *Gallus;* bone structure of *Balaenoptera;* tumors of the mammary gland of mice. Moreover: root cells of *Allium:* the *Ciliata: Isotricha, Opalina* and *Paramaecium;* eggs of *Ascaris, Limnaea* and *Cyanea;* nematocysts of *Corynactis.* Fig. 2, representing part of an intestinal cell of *Ascaris,* shows the striated border and beneath that the plasma zones with mitochondria. Fig. 3 shows a section of a nematocyst of the sea anemone *Corynactis.* The windings of the spirally rolled canal in the capsule have been cut laterally. The capsular wall is seen to consist of fibrils about 10 mμ thick, which run parallel to the longitudinal axis of the capsule. These fibrils show a very regular transverse striation with a period of 280 A.U.

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ON THE USE OF THE ELECTRON MICROSCOPE IN WOOD ANATOMY.

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I. Introduction.

The development of a new replica technique by Ir A. J. A. Nieuwenhuys (1948) (Division for Electron Microscopy of the Technical Physics Department, Delft), makes possible to prepare specimens from porous materials.

In this way the original structure of wood can be investigated with the aid of the electron microscope, whereas so far attention was mostly given to isolated fibres. Whether this new technique can be of any use in wood anatomy and if so, which line of investigation might yield the greatest profit, is studied in cooperation with the Division for E.M.

II. Materials and methods.

The material comprised clear cut end surfaces and longitudinal surfaces of normal spruce blocks *(Picea excelsa* Lk.) and normal pine blocks *(Pinus silvestris* L.).

Moreover spruce samples, decayed under laboratory conditions for a period of 3 months by *Coniophora cerebella* Alb. et Schw., strain Pless, were examined. From half of the blocks the loss in weight when dried at 105° C. was determined (varying from 23,3 % to 32,3 %). The other half was dried at room temperature and these parts were investigated.

The cellwall structure in the line of failure in pine samples, compressed parallel to the grain, was also studied.

So far experiments are done with "uncleaned" wood, which means that no chemical constituents (such as lignin) have been removed.

Nieuwenhuys' technique as used for the present investigation includes the following successive operations:

1. A print of a solid wood block is made with alkathene (polyethylene) grade 20, at 115° C and 2 atm. pressure.
2. A positive collodion replica is made of the alkathene matrix. As this replica cannot be prepared thin enough to be used directly in the E. M. two more steps are necessary.
3. An aluminum film of about 20 A.U. thickness is evaporated in vacuo from all sides on the collodion print. To obtain a relief in the picture a gold manganin shadow cast is imposed on the aluminum film at an angle of 45°.

4. The collodion is dissolved in the condensing vapour of amylacetate.

Specimens have been examined with the aid of the Delft electron microscope.

III. Fields of application.

A. Application to problems of identification and cellwall structure.

It may be expected beforehand that the possibilities of the electron microscope in identification problems will be limited. The handlens method and the usual microscopical technique reveal a larger area than the electron microscope and with less preparation. In wood identification the arrangement, grouping and dimensions of the elements play an important role and exactly for these characteristics a larger view is necessary. It is true that the electron microscope allows greater enlargements than the methods just mentioned, but it only shows details of the structural elements of wood. The preparation techniques are much more complicated than the methods used so far. The development of such techniques may be important if differences between nearly akin species are looked for and also if details are wanted of structural characteristics which are insufficiently revealed by the common microscope. In this relation the author refers *e.g.* to vestured pits, the structure of which was explained wrongly in the beginning. Some micrographs give an impression of the results which may be obtained by studying the structure of pits with the aid of an electron microscope. An example is given in Fig. 1. The investigation of the normal cellwall structure can be highly stimulated by a well developed technique. Special attention is

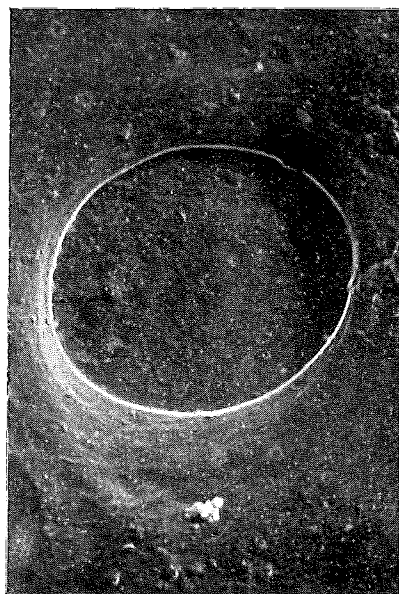

Fig. 1. Replica of simple pit occurring on the area of contact between a ray-cell and a fibre tracheid (cross-field) in normal spruce *(Picea excelsa* Lk.). In the secondary wall adjacent to the pit a more or less spirally arranged striation is visible. 6.500 x

payed to the direct observation of thin layers of the material by a high power microscope (400 kV) such as now in operation at Delft. The relative advantages of sections as against replicas should be investigated.

B. Investigation of the structure of decayed wood.

The electron microscope will offer greater possibility as to the investigation of timber decay than in wood identification. A high resolving power applied to a small area is wanted here. This applies *e.g.* to places where fungal hyphae pierce the wall. Changes occuring in the cellwall under these circumstances are of interest as well as the structure and the deformation of hyphae.

It can be expected that early stages of decay in the cellwall may be observed, which do not show up with the aid of an ordinary microscope. These problems are illustrated with some micrographs, the interpretation of which is still quite uncertain. An example is given in Fig. 2.

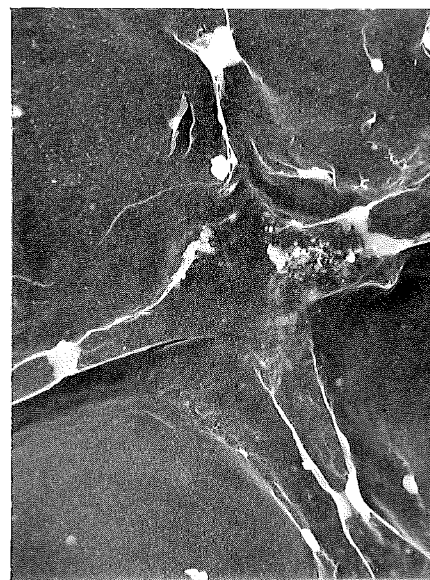

Fig. 2. Replica of cross section of fibretracheids in spruce, decayed by *Coniophora cerebella* Alb. et Schw. Four cellwals coming together show white areas. These consist of extra material which allows less penetration of electrons than the rest of the replica. From other pictures it appears that these areas and particularly constrictions as shown in the upper wall precede a local breakdown of the walls. As these features are not observed in normal or mechanically damaged wood, they seem to be typical for fungal attack. 1.800 x

The electron microscope can be used to investigate which part preservatives play in preventing decay of wood. The penetration and adhesion of these preservatives might become a subject for study.

C. Investigation of mechanically damaged wood.

Photographs of changes in the structure of the cellwall as a consequence of mechanical deformation may reveal the influence of different types of damage, (*e.g.* compression, shock and so on). Such material may also provide an opportunity to study the normal structure of the cellwall. The occurence of slip planes, on the interpretation of which many discussions are known in literature, can be demonstrated. This subject was investigated several times with the aid of the electron microscope but, as far as the author knows, only on isolated fibres. Slip planes occuring in tissue structure may show

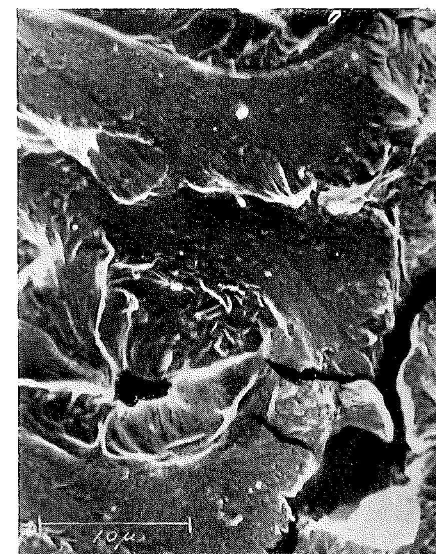

Fig. 3. Replica of cross section through fibre-tracheids in the line of failure of a pine sample *(Pinus silvestris* L.), compressed parallel to the grain.

Note the granular structure in the cellwall (*e.g.* in the left hand upper corner of the picture). This feature does not show up in micrographs of normal and decayed wood and therefore seems to be characteristic for mechanical damage.

gradual transition stages and should be investigated as well as damaged isolated fibres. Using the punch technique, we were able to give a precise location of the sample. Changes of structure observed in mechanically damaged wood (shearing) are illustrated with some micrographs, an example of which is given in Fig. 3.

Micrographs of each of the subjects mentioned, show mutual resemblance. However, obvious differences are noticed between the groups "normal structure", "decayed wood" and "mechanically damaged wood". Further investigations will show whether these differences are really characteristic or whether they are artefacts. The interpretation of the photographs is by no means certain in this respect. More material and photographs are needed to solve this problem.

The object of this preliminary communication is to contact investigators who are interested in an electron microscopic study of wood anatomy. It would be desirable to exchange information among those who collected data in this field. The author suggests wood identification, decayed and mechanically damaged wood as possible subjects of investigation.

On a recent visit to the U.S.A. (June 1949) the author had an opportunity to compare results of the present study with those obtained by Prof. R. W. G. Wyckoff (National Institute of Health, Bethesda), Prof. B. Grondahl (School of Forestry, Seattle) and Mr Wiegand and Mr Fischbein (Prof. R. A. Cockrell's division in the School of Forestry, Berkeley, Calif.).

From these contacts it appeared that close team-work in the study of wood anatomy with the electron microscope will benefit the results considerably. Such cooperation also will give to all investigators concerned a deeper insight in their field of interest.

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LA FORME DES ESPACES SUBMICROSCOPIQUES.

C. A. BAUD, Institut d'Histologie, Faculté de Médecine, Lyon.

Il est difficile, lors de l'examen des objets biologiques au microscope électronique, d'apprécier les véritables dimensions et la véritable forme des espaces submicroscopiques (c.à.d. des espaces qui séparent les uns des autres les éléments structuraux submicroscopiques). En effet, les méthodes habituelles de préparation (dissociations, etc.) altèrent considérablement les rapports spatiaux entre les éléments. Le but de cette communication, purement technique, est de présenter deux méthodes d'étude des espaces submicroscopiques au microscope électronique: la méthode des moulages, et la méthode des coupes après inclusion dans un milieu approprié.

1. La méthode des moulages.

Cette méthode (C. A. Baud [1]) s'inspire de la technique industrielle de la charge de la soie. Elle consiste à précipiter une substance minérale à l'intérieur des espaces submicroscopiques de l'objet, puis à détruire celui-ci par micro-incinération selon le procédé de A. Policard [2]; on obtient ainsi un moulage minéral des espaces submicroscopiques.

La charge à l'étain, qui nous a donné satisfaction, est celle habituellement utilisée dans l'industrie, depuis les trauvaux de R. Gnehm et E. Bänziger [3]. L'objet (par exemple fils de soie) est placé d'abord dans une solution de tétrachlorure d'étain, puis lavé longuement à l'eau, et traité par une solution de phosphate disodique; plusieurs passages successifs dans cette série de bains assure une charge maxima; on termine par un bain de silicate de sodium. La nature du précipité formé dans les espaces submicroscopiques fait encore l'objet de discussion; il semble s'agir d'oxyde d'étain ayant adsorbé du phosphate et du silicate. L'examen de l'objet chargé, par la méthode de diffraction des rayons X, montre que le précipité se trouve sous forme de microcristaux disposés sans orientation dans les espaces submicroscopiques (R. O. Herzog et H. W. Gonell [4], et W. Stockhausen [5]).

Si la préparation chargée est soumise à la micro-incinération, toute la matière organique disparaît; il reste un moulage minéral des espaces submicroscopiques. De tels moulages conservent l'aspect microscopique de l'objet, et présentent une biréfringence de forme correspondant à sa texture.

De petits fragments de ces moulages sont examinés au microscope électronique. Ainsi est obtenue une image de la forme et de la taille des espaces submicroscopiques de l'objet; de telles données peuvent être

comparées à celles obtenues indirectement par l'étude de la biréfringence de forme.

2. La méthode des coupes.

Cette méthode utilise l'inclusion dans le methyl-acrylate de méthyle, suivant le procédé de J. Coudert et C. A. Baud [6, 7]. Le fragment d'organe à examiner est fixé de la manière habituelle, deshydraté par l'alcool, imprégné par le toluène et enfin par le méthyl-acrylate de méthyle monomère pur. Le monomère est ensuite polymérisé à l'étuve à 56° C pendant 24 heures, en présence d'une faible quantité de peroxyde de benzoyle comme catalyseur. Au sortir de l'étuve, l'objet se trouve inclus dans un bloc dur de résine synthétique.

Le milieu d'inclusion a pénétré parfaitement jusque dans les espaces submicroscopiques de l'objet. Si en effet on détruit alors celui-ci par un réactif approprié n'attaquant pas le méthyl-acrylate de méthyle, il reste dans la masse un „fantôme" ayant conservé son aspect. Cet aspect est dû au méthyl-acrylate de méthyle déposé dans les espaces submicroscopiques de l'objet, et resté en place lors de sa destruction. Un tel „fantôme" montre une biréfringence de forme topographiquement identique à celle de l'objet, ainsi que l'ont montré C. A. Baud en M. J. Dallemagne [8].

L'excellente pénétration, dans les espaces submicroscopiques, de cette masse douée de propriétés mécaniques très intéressantes, nous a conduit à l'utiliser comme milieu d'inclusion pour les coupes ultra-minces destinées à l'observation au microscope électronique. Nous avons en effet obtenu ainsi, en utilisant un microtome Spencer type 820 modifié suivant les indications de D. C. Pease et R. F. Baker [9], des coupes de quelques dixièmes de micron d'épaisseur, sans aucune des déformations de l'objet qui se produisent lors de la confection des coupes avec les autres masses d'inclusion.

Les coupes sont collées sur lame de verre; la lame est ensuite plongée dans l'acétone ou le dioxane pour dissoudre le méthyl-acrylate de méthyle, puis dans une solution de Rhovinal (ou Formvar) dans le dioxane, et sèchée; le film de Rhovinal contenant les coupes est ensuite prélevé de la manière habituelle, et monté sur les porte-objets du microscope électronique.

Ce procédé simple fournit, grâce aux propriétés de la masse d'inclusion, une préservation satisfaisante des relations spatiales entre les éléments constitutifs de l'objet.

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Discussion.

Prof. Gabor: How can you be sure that it is „biréfringence de forme" and not „biréfringence de structure".

Answer: La biréfringence de forme dépend de l'indice de réfraction du milieu d'imbibition, et se distingue en cela de la biréfringence intrinsèque (ou biréfringence de structure). Dans nos observations sur les moulages minéraux et les moulages en méthyl-acrylate de méthyle, nous avons constaté les variations de la biréfringence en fonction des indices de réfraction des liquides d'imbibition.

ELECTRON MICROSCOPY IN RELATION TO THE MEDICAL SCIENCES.

S. MUDD, Philadelphia, Pa., U.S.A.

"L'infinité de la petitesse", in the words of Blaise Pascal, includes extensive areas whose submicroscopic dimensions render them inaccessible to light microscopy but which are accessible to exploration with the electron microscope. It is the purpose of the present paper to survey very briefly these areas within the medical sciences.

The region of fine dimensions which is inaccessible to light microscopy but accessible to electron microscopy has already been shown to comprise structural details of bacteria, rickettsiae, viruses and bacteriophages, the fine structure of protoplasmic fibrils and fibrous structures, macromolecular-scale structure within appropriately prepared tissue culture cells, the submicroscopic organization of liver, nerve, muscle and other tissue and of chromosomes, chloroplasts, mitochondra and colloidal molecules and particles. Electron microscopy on the American continent is only about ten years old; with advancing techniques even wider prospects will unquestionably open up, in particular to use of the electron microscope in parallel with longer known research instruments and procedures.

In all bacteria cell wall and inner protoplast with limiting plasma membrane, and in various species extracellular capsules, flagella, and developing and mature spores, have been clearly visualized. The bacterial

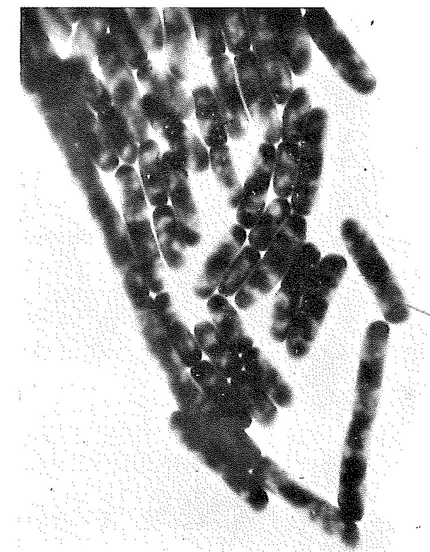

Fig. 1. A microcolony of normal *Escherichia coli* cells after 3½ hours' growth at 37° C on thin collodion film overlying 1.5 % extract agar in a petri dish. Double objective lens used in making the electron micrograph. There is good correlation between the light areas and the nuclei demonstrated by light-microscopic technique.

nucleus, and even macromolecular scale fine structure within protoplast, cell wall and spore coat have been visualized also, albeit still with technical problems as limiting factors. Certain bacteria have long been known to possess the remarkable biochemical capacity of synthesizing living protoplasm from the simplest source materials; such bacteria are now shown to be morphologically complete cells with characteristics convenient for direct electron microscopic investigation of the structure and organization of protoplasm in this relatively simple form.

Antibodies have been made visible in combination with the surface structures of bacteria and viruses. Antibodies combine chemically with and form surface deposit on cell wall and flagella of non-capsulated bacteria and on the surfaces of virus particles. In the case of virulent pneumococci, antibodies and other serum proteins permeate and swell the extra cellular capsule.

Certain germicides may readily penetrate the bacterial cell wall and attack the plasma membrane. Antibiotics may inhibit cell division and result in morphological aberrations or in bacteriolysis. Morphological difference in the nucleus in phase variants has already been reported. Study by light and electron microscopy of the bacterial nucleus in relation to genetic change and to the action of mutation-inducing physical and chemical agents is a fascinating problem to the threshold of which current advances in the cytology and genetics of microorganisms and the techniques of electron microscopy have just brought us.

The electron microscope has made a peculiarly valuable contribution to the study of the rickettsiae and viruses, which are near or beyond the limits of resolution of the light microscope. The rickettsiae, viruses of the lymphogranuloma-psittacosis group, and the pox viruses have been shown to have the structure of very simple cells equivalent to that of tiny bacteria. The cell wall and protoplast may be differentiated, and in vaccinia there has even been described what appears to be a nucleus. The influenza virus, which is quite invisible with the light microscope, has also apparently a surface membrane and inner protoplasm. The particles of bacteriophage, which are often considered as viruses parasitic upon bacteria, are usually tiny sperm-shaped particles containing a head of characteristic shape and a tail. The head of coliphage particles is of complex structure in that it contains a characteristic pattern of granules. The contents of the head, moreover, may be released and leave an empty envelope with tail attached. The currently available evidence strongly suggests that the larger viruses, such as those of lymphogranuloma and psittacosis and of the pock diseases, multiply like bacteria by division, but that the most thoroughly studied bacteriophage particles are synthesized within the bacterial cell. The fact that phage-infection of a bacterial cell imposes a new metabolic pattern on the infected cell and results in the elaboration of multiple new infectious phage particles is a remarkable one, study of which may well afford clues to the understanding of virus-induced neoplasia. Colony-like groups of virus particles have been demonstrated in the cytoplasm of cells of virus-induced neoplasia.

Isolated virus particles under proper conditions have been shown by Wyckoff to form beautiful crystalline arrays in three dimensions. In general it may be said that the problem of visualizing any of the viruses is primarily one of isolation and preparation. All can be clearly demonstrated by the electron microscope once they are isolated.

Protoplasmic fibrillar structures have lent themselves particularly well to structural analysis by the electron microscope in parallel with studies by polarized light, X-ray diffraction and other physical and chemical means. Beautiful periodic regularities in ultrastructure have been demonstrated by Schmitt, Hall and their collaborators in connective tissue, muscle, and nerve fibrils and their component proteins. A foundation knowledge of normal ultrastructure is being laid for later comparison with ultrastructure in such pathological conditions as the collagen diseases, and nerve and muscle inflammations and degenerations.

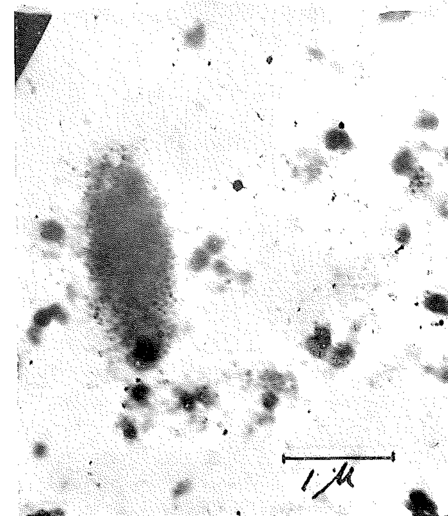

Fig. 2. A cell of *E. coli* completely packed with T_2 bacteriophage. Such cells are frequently found in preparations examined 18—20 hours after inoculation with the phage.

The scope of useful applications of the electron microscope in the medical sciences has been broadened steadily by the development of new techniques both preparative and instrumental. Tissue culture cells, their components and inclusions are being rendered accessible to investigation through techniques under development by Claude and Porter. Regularities of internal structures of muscle, collagen and nerve fibrils have been revealed through adaptation of fixation, "electron staining" and shadowing procedures. Metallic shadow casting has increased contrast and has made possible the imaging of minute structures in three dimensions. Techniques of surface replica which have had wide application in metallography, mineralogy, *etc.* are being applied to the study of biological surfaces.

Incomparably the greatest broadening of applications of electron microscopy has been made possible by the elaboration of methods for cutting thin sections of tissue. Early work by O'Brien and McKinley has been followed by the construction of a high speed microtome capable of cutting sections of the order of 0,1 μ in thickness. Studies of such sections of various tissues including neoplasia are appearing. Most recently D. C. Pease and R. F. Baker of the University of Southern California, employing double imbedding in collodion and paraffin and a special adapter to the conventional microtome have cut and made beautiful electron micrographs of sections of liver, muscle, nervous tissue and chromosomes only 0.05—0.10 μ in thickness. This technical achievement makes accessible to electron microscopic study the enormous field of the fine structure of normal and pathological tissues.

Because of the newness of electron microscopy, the high cost of the instrument and the expertness required for proper maintenance, operation and interpretation, the electron microscope is certainly not now, nor is it likely to become soon, an office instrument. A beginning has been made, however, in application of electron microscopy to the differentiation of certain related viruses. Such uses and the newly developing techniques of preparing tissue sections will probably soon give electron microscopy areas of application in diagnostic laboratories in the larger hospitals and institutes. The prospect which is most exhilarating, however, is that of pure scientific research, the exploration of one, possibly two new orders of magnitude of fine dimensions by electron microscopy supplementing other current research instruments and techniques.

SOME OBSERVATIONS ON BACTERIAL FLAGELLATION.

Miss W. VAN ITERSON, T.P.D., E.M.Div., Delft.

This paper is the outcome of a collaboration of two laboratories: R. C. A. Laboratories at Princeton N. J. where the authoress had the privilege of spending some time, and the Institute for Electron Microscopy at Delft where Dr A. L. Houwink carried out a similar investigation. The object of the study was a re-investigation with the electron microscope of the problem of bacterial flagellation, the discussion of the respective characteristics having again become topical, in particular after the introduction of the study in the darkfield of the light microscope.

Techniques of preparation are of special importance since they have to stand out against criticism from the side of bacteriologists. It has been claimed that the flagellation of bacteria when studied after evaporation of the water in which they have been suspended is an artifact. By currents in the water the flagella are said to detach and secondarily to reattach to the cell body. However, when bacteria floating in distilled water or in a solution of a fixative are carefully picked up on a collodion film, such preparations when compared with those made according to a more reliable method, *e.g.* stripping-off from an agar surface, as a rule yield the same results. Dr Houwink has successfully applied the method of stripping-off bacteria also to bacteria previously developed in liquid culture. Dr Hillier rightly questions the reliability of the stripping-off technique in all cases, because the preparations are brought into touch with a water surface. Therefore Hillier, Knaysi and Baker introduced a method of growing bacteria on top of a collodion membrane spread over nutrient agar in a petri dish. Thus, when the film is floated off,the undesirable contact of the preparation with the water is avoided.

With the first mentioned methods it was possible to put the actual existence of peritrichy and cephalotrichy beyond doubt, and other interesting data could be obtained. In young cultures flagella turned out to be less numerous and shorter as compared with older cells. With the method of growing bacteria on top of the collodion film these observations have been confirmed and it could be established that for the development of flagella the motility of the organisms is not an essential condition. A striking picture has been obtained from *Escherichia coli,* in which after 4 hours' development a few flagella were present, but these were very straight and much shorter than those in older cultures

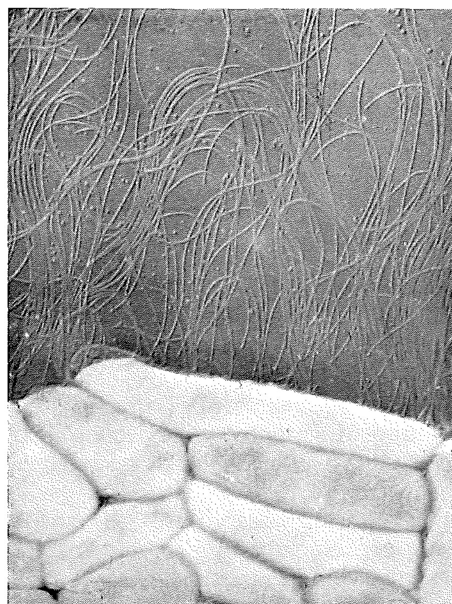

Fig. 1. After 6 hours' incubation on collodion spread over nutrient agar *Proteus vulgaris* developed flagella. These bacteria probably grew in a relatively large amount of water, which caused them to shift together during desiccation.
Made at R.C.A. Lab.

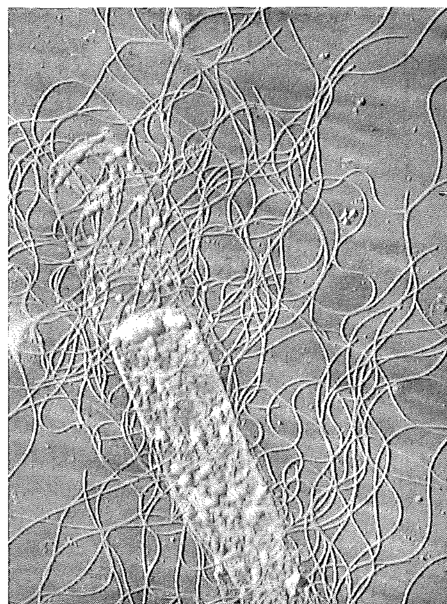

Fig. 2. *Proteus vulgaris*. Preparation Dr C. F. Robinow. In the upper part of the autolysed swarmer cell nothing of the protoplast is left except for the granules at the base of the flagella. 11 000 x.
Made at R.C.A. Lab.

where they are undulated. Therefore the flagella of the particular cell were considered to represent "juvenil flagella", which evidently grow out individually and in peritrichous arrangement.

It must be considered impossible to explain flagella as being secondary products of an irreversible untwisting of a slimy tail. * This slime theory is neither consistent with the results obtained by germination on the collodion membrane of spores of *Bacillus mesentericus*. In micrographs of the latter it was possible clearly to distinguish the flagella, having a linear structure, from the simultaneously present amorphous slime.

Following a suggestion of C. F. Robinow a study was made of the implantation of the flagella in the swarmer cells of *Proteus vulgaris* after their autolysis. In the best preparation nothing of the original protoplast remained except for a number of rather uniform spheres of about 100 mμ from which the flagella are seen to arise. Fig. 2.

Flagella thus turned out to represent definite organs of the bacteria. Their presence and arrangement can well be applied in the diagnosis of bacterial species and in their classification. They grow out individually and emerge — at least

* N.B. cf Houwink, van Iterson, Biochim. Biophys. Acta, in press.

in the case of *Proteus vulgaris* — from basal granules. A relation has to be sought between these results and the finding of a tail on many actively moving bacteria. In several instances it could be noticed in electron micrographs that flagella possess a tendency to unite into bundles. An extreme case of this tendency is to be found in what the Germans have called a "Riesen Zopf". Pictures of flagella aggregations call to mind those of aggregates of fibrous proteins belonging to the keratin-myosin- epidermis- fibrinogen group. In the light of Weibull and Astbury's finding that *Proteus* flagella represent a comparable protein this seems of importance. This tendency to aggregate as well as the remarkable observation of the presence of basal granules may prove to be helpful in a future explanation of the function of flagella.

Discussion.

Dr Kellenberger shows a micrograph of a cell of a non-flagellated strain of *Bac. subtilis*. The cell has been broken by centrifugation. Filaments which look like flagella appear. The same happens with many other treatments *e.g.* with distilled water, and after sporulation. It may be that one type of flagella is a continuation of internal structures through the membrane.

Answer: I do not think it will be necessary to add much to Dr Kellenberger's statements. His treatments of bacteria are quite different from ours, nevertheless there seems to be a close resemblance between his filaments and our flagella. It may well be that his experiments offer further proof that flagella are definite bacterial organs of internal origin. This, however, is difficult to judge at first sight.

Dr Cosslett: Do micro-flagella exist? We have found some fine fibrils, along with one major flagellum. In this case the minor fibrils may very well be metabolic extrusions. Also we found one case in which the flagellum showed two or three branches. Possibly the flagellum is made up of these finest fibrils.

Answer: Although I should be glad to enter into this question I would suggest to await Dr Houwink's paper in which much attention will be paid to the occurrence of threads (filaments), other than flagella on bacteria.

ELECTRON MICROSCOPY OF INFLUENZA VIRUS.

I. M. DAWSON and W. J. ELFORD, National Institute for Medical Research, London

Since any new technique which will make visible a hitherto invisible disease-producing agent is of immediate value in virus research it is easy to see why electron microscopy has been applied from a very early date to the study of influenza virus. The early work of the American schools [1, 2] indicated the virus particle to be entirely round in form and about 100 mμ in size and later Wyckoff and Moseley [3] noted the presence of filaments in purified preparations of influenza virus. The technique used in the investigations here reported has been evolved from the original experiments of Hirst [4] and McClelland and Hare [5] who noted that agglutination of red blood cells occurs when in contact with influenza virus, through adsorption of the virus.

The first investigations of the physical picture given by virus adsorbed on the surface of red cells were undertaken independently by Heinmets[6] and by ourselves. Heinmets has produced micrographs showing the physical adsorption of virus to the surface of red blood cells, but the difficulties encountered through the elution of the unfixed virus from the intact cell and the opacity of the latter made quantitative work impossible with his technique. We have concentrated on the study of the adsorption of virus on to fowl cells, laked by saponin (Dounce and Lan [7]). The laked-cell membrane is sufficiently thin for transmission microscopy. The virus can be adsorbed on the laked cells which are then washed in buffer on the centrifuge followed by fixation in osmic acid — a critical part of the procedure since it prevents elution of the virus during the subsequent washing in distilled water. This method has been of the greatest value to us in recent studies of the morphology of influenza virus since we have found that it is possible to examine virus, adsorbed directly from allantoic fluid, without any lengthy purification procedure. (See Dawson and Elford 1949 [8]).

The classical strains of influenza virus, the PR8 strain of influenza A shown in Fig. 1 and the Lee strain of influenza B have a characteristic morphology. Although a few filaments are seen on the field the large majority of virus particles are characteristically spherical. This picture illustrates the pattern of the adsorption process. No regular packing of the virus on the cell surface can be seen suggesting that there is no obvious geometric pattern of receptors on the surface of the red blood cell.

We found that filaments appeared in much greater numbers in recently isolated strains of influenza than in the classical laboratory strains of the virus. This is illustrated in Fig. 2 which is a micrograph of a preparation of influenza A/Paris/Lépine.

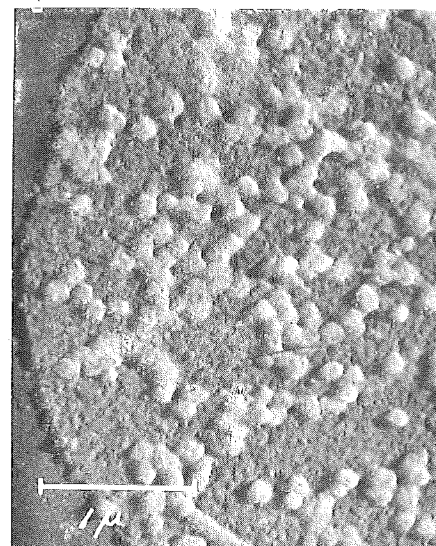

Fig. 1. Influenza (PR8) adsorbed on the membrane of the laked fowl red cell. Shadowed with palladium. Micrograph reproduced by courtesy of the Editor of Lancet.

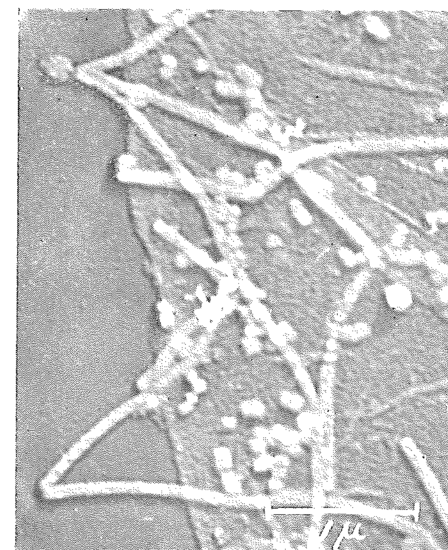

Fig. 2. Influenza A/Paris (PL1)/49 adsorbed from infected allantoic fluid.

The whole subject of the morphology of influenza virus is thus thrown into sharp relief by the pictures of these recently isolated strains. Questions immediately present themselves as to whether the filaments are true forms of the virus. Or, are the filamentous structures some fibrous components released from the infected host cell? Or again, are we faced with the situation where we have a virus and symbiant? Let us marshal the evidence so far available to answer such questions.

1. The long forms are not electron microscope artefacts since they are visible in the untreated fluid when examined in the ultra-microscope.
2. No filaments have been adsorbed directly from normal as distinct from infected egg allantoic fluids. Heinmets and Golub [9] have shown that fibrillar structures can be derived from normal allantoic membrane cells. These exhibit a 64 mμ banded structure such as is shown by collagen. Our influenza long forms do not possess this banded structure, although internal beading is often in evidence. Experiments with collagen and fibrin gave no indication of adsorption of these proteins on the laked red blood cell surface.
3. The filaments and round bodies are both adsorbed and eluted from the red cell surface in exactly the same way. Furthermore, both forms of the virus which after heating to 56° C for ½ hr. can still be adsorbed but no longer elute spontaneously, are removed from the cell surface by treatment with homologous antiserum.
4. Under the cardioid ultramicroscope the long forms may be observed to have become aggluti-

nated when mixed with the appropriate homologous antiserum. No success has so far attended our attempts to cultivate the virus *in vitro*. Examination of virus after as many as 30 consecutive passages in eggs and after 24 passages in mice followed by one in eggs, has still revealed the presence of both filamentous and round eluting bodies.

Virus has been filtered through the tightest gradocol membrane likely to permit passage of the round forms of the virus and the filtrate when inoculated into eggs has yielded both long and round forms, which suggests that the elementary body can give rise to the elongated forms when grown in a suitable environment. The effect of the time of growth of the virus in the egg on the morphological picture was also studied and material adsorbed 48, 72 and 120 hrs. after inoculation showed a progressive decrease in the number of long forms present. In this connection our findings are a complete contradiction of a statement made by Heinmets that long forms of influenza appear to be aggregates produced by storing the virus for a prolonged time.

Evidence so far accumulated indicates a close relationship between the round and filamentous structures manifested by certain recently isolated strains of influenza. This intimate relationship is seen both in conditions requisite for their development and also in their surface properties. While the round elementary body is undoubtedly the fundamental virus unit, its multiplication to yield the diploids, short chains, rods and filaments is apparently a characteristic feature of certain recently isolated strains when passaged amniotically or allantoically in fertile hen's eggs.

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Discussion.

Dr Lépine: During the recent influenza epidemic we have isolated in Paris ten different strains of influenza virus.

We have found filamentous forms, and I agree with Dr Dawson that these forms are in definite relation with the virus.

One fact has raised our attention: our strains show a wide variation in their antigenic properties, going from pure A strains to pure FM1 strains. It so happens that strains that have shown *no* filamentous forms were A strains, while those with thread-like particles were FM1 strains. So we believe that the filamentous particles *might* represent the FM antigen. But this should be substantiated by more numerous observations than ours which bear on only ten strains.

Answer by Dr Dawson: Certainly the relative proportion of the elongated forms to the elementary round bodies does vary from strain to strain and even from passage to passage. In our experience the recently isolated 1949 strains, passaged in eggs, have been characterised by a high proportion of the filamentous forms in contrast to the classical A strains which, after many laboratory passages, now show predominantly the round elementary bodies. Newly isolated strains which have contained but few rod-like forms have invariably proved to be 'B' virus. Many A strains dating as far back as 1937, in addition to the A'strains 1946-48, have been examined and we find they contain, in varying proportion, rod-like and filamentous structures in addition to the fundamental round elementary body.

ELECTRON MICROSCOPY OF MURINE PSITTACOSIS VIRUS.

C. F. BARWELL, London Hospital; I. M. DAWSON and A. S. McFARLANE,
National Institute for Medical Research, London.

Mice were infected intraperitoneally with a strain of psittacosis received from Professor Bedson. At death, which usually occurred in 48 hrs., the spleens were removed, disintegrated in saline and the virus purified by repeated differential centrifugation in this medium. A stage is soon reached beyond which no further improvement in the purity of the specimen is achieved by repeating the centrifugation. The

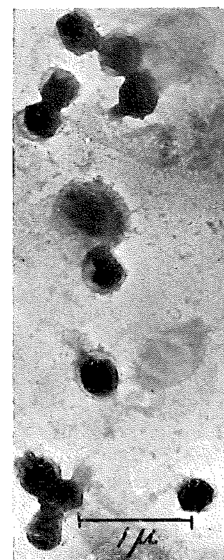

Fig. 1. Transmission micrograph of centrifugally purified psittacosis virus.

preparation so obtained does not consist solely of virus bodies, although a few can always be found in the electron microscope field which are relatively free from cellular debris (Fig. 1). In similar conditions much better purification is obtained, for example, of vaccinia virus from infected skin. This is probably due to the fact that the proportion of virus to normal tissue in the whole spleen is much less than in the superficial scrapings of the infected epidermis. There is therefore a higher probability of the splenic virus being contaminated with cellular fragments of about its own size.

Treatment of the virus deposit with molar sodium chloride causes a marked reduction in its naked-eye bulk, and after washing to remove the last traces of sodium chloride much better electron micrographs are obtained. In particular, the background is cleaner, the amount of debris is reduced and the edges of the virus bodies are sharper (Figs. 2 and 3).

Psittacosis bodies consist of a dense central mass with wrinkled or indented surface, the whole being enclosed in an envelope which appears at the edge as a flattened membrane. The length of the shadow

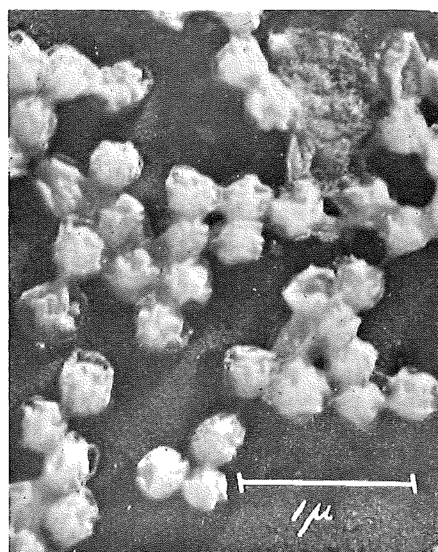

Fig. 2. Palladium shadowcast micrograph of psittacosis virus further purified by treatment with molar sodium chloride.

indicates that the centre is raised by nearly a diameter. The mean size is 361 m μ with a standard deviation of 42 m μ.

Large forms have been recognised for many years in stained preparations [1]. However, in these experiments use was made almost exclusively of material from mice dying acutely of psittacosis where elementary bodies are abundant and few larger forms seen in stained smears. In a few tests where infected spleens were taken 16 hours after infection in order to demonstrate large forms unsatisfactory results were obtained owing to the difficulty of purifying the much smaller quantity of virus present at this stage. A few micrographs, however, showed large forms about 800 mμ in diameter (Figs. 3 and 4) having the same general characteristics as the smaller ones. In both groups there is a wide variation in appearance of the bodies from wrinkled spheres to almost empty flattened sacs.

It is not possible to say how much of the irregular contour of psittacosis virus is due to drying preparatory to electron microscopy. A similar appearance is seen in electron micrographs of the Seiffert sewage organism [2] and in the rickettsiae [3]. It is also found in psittacosis cultivated in the embryonated egg [4, 5] and in a related virus, namely feline pneumonitis [6]. The simplest explanation of the available evidence appears to be that the native virus is highly hydrated and has a relatively thick outer membrane which tends to collapse in folds on drying. Appearances especially of some of the larger forms suggest that the membrane is rigid enough to remain raised off the underlying film in one or two places without support from below. Whereas some bacteria show

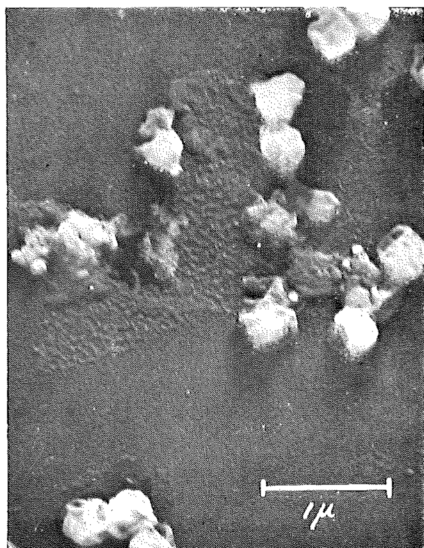

Fig. 3. Virus preparation purified as in Fig. 2. In the group of three virus particles visible on the extreme left of the field the central particle is larger and flatter than the average.

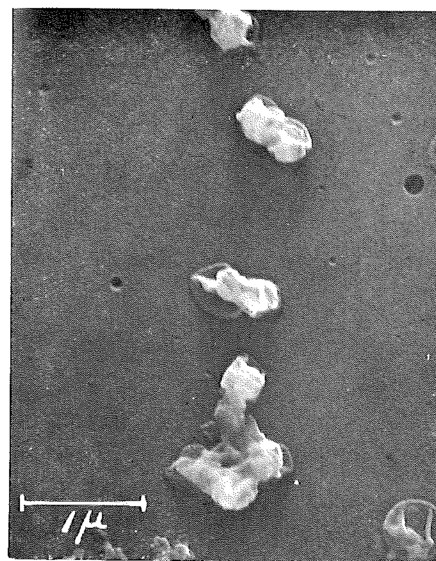

Fig. 4. Preparation purified by treatment with molar sodium chloride and digested with pepsin at pH 3. A considerable reduction in the amount of associated debris is apparent. A large flattened virus body is visible at the bottom right hand corner of the field.

similar relatively empty forms, in these the raised portions of the cell membrane are obviously supported by shrunken masses of cytoplasm.

No significant change could be seen in the electron micrographs after adjusting the pH of the virus to 3 with hydrochloric acid or after incubating this acidified virus with pepsin (Fig. 4). A few bacteria which we have examined, for example *B. proteus, Staph. aureus,* are also unchanged by this treatment [7]. However, in the same conditions a dramatic change takes place in vaccinia virus [8] with loss of at least three-quarters of the virus substance into solution. The behaviour of psittacosis virus towards pepsin is consistent with the view that the outer membrane is impermeable to this enzyme.

All the evidence available including the wider size variation tends to differentiate psittacosis, and indeed all the members of the so-called psittacosis-lymphogranuloma-pneumonitis group, sharply from the rickettsiae or microbacteria generally.

There is a remarkable morphological similarity between this virus and that of feline pneumonitis as shown by a comparison of our pictures with those of Hamre *et al.*

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Discussion.

Dr Lépine: When Dr McFarlane was showing his beautiful electron microscope pictures of the Psittacosis virus, I was struck by their absolute similarity with those of the lympho-granuloma Nicolas Favre virus, which we have published some time ago with Mlle Croissant. In fact, some of these pictures were taken in Delft.

Dr McFarlane is thus providing us with a new proof of the very close relationship that exists between the two viruses, as previously examplified in the studies which we have devoted with Mlle V. Sautter on the thymonucleic acid content of both the Psittacosis and L.g.v. particles and as shown since by many studies on their respective and common antigenic properties by various authors.

I wish also to point out that the Virus Division of the Institut Pasteur had, too, to consider the hazards involved in the manipulation of virulent specimens intended for electron microscope examination. We have

overcome this real danger with a short exposition (30 seconds) to ultraviolet radiations of all mounted specimens: this is largely sufficient to inactivate thin films of virulent material without alterating their morphological aspect. So we have made a rule that no specimen should leave the inoculation room or the sterile cubicle used for handling infected material without being passed in the beam of a fluorescent tube of the so-called "germicide lamp" type for a period of time sufficient to inactivate it. This method has so far proved to be very satisfactory.

Dr Dekking: Has Kurotchkin's observation of the difference in morphology between boiled and unboiled suspensions been confirmed in view of the elucidation of antigenic structure?

Answer by Dr Dawson: Kurotchkin *et al.* reported that, in the case of human pneumonitis virus, the outer zone of low electron absorbing power could be removed by heating for five minutes in a boiling water bath: the effect is not illustrated nor is it described for the other viruses which they studied. We did not investigate the effect of heat on the morphology of psittacosis virus but failed to find any such alteration as the result of treatment with dilute acid which resembles heating in so far as it produces the same change in antigenic behaviour.

AN ELECTRON MICROSCOPE STUDY OF RAM SPERMATOZOA.

Miss M. H. G. FRIEDLAENDER and J. T. RANDALL,
Physics Laboratory King's College, London.

Mammalian sperm show characteristic differentiation into a head, linked by a "neck" to the middle-piece which, in turn is followed by the principal tail part and the short tail end.

Methods of preparation.

For routine fixation, formalin and osmium tetroxide vapour were used; phosphotungstic acid, too, proved an efficient fixative. Osmium tetroxide was used for staining, both as liquid and vapour, and showed up interesting head structures. The staining action of phosphotungstic acid was uncertain.

Head.

The dimensions of the head are approximately 8 μ, 4.25 μ, and $\backsim 1 \mu$ for length, maximum width and thickness respectively. Shadowing with palladium and palladium-gold showed up considerable surface detail. Starting with the outermost layer, the fibrous galea capitis covers the anterior part of the head. A sharply defined equatorial segment corresponds to the rather vague and larger equatorial zone of unshadowed preparations. The upper limit of the segment is marked by the acrosome cap, and its lower by the nuclear ring. The segment surface is smooth, and closely attached to an underlying layer which appears to be covering the entire head. Experiments with ultrasonics helped considerably in clarifying these points.

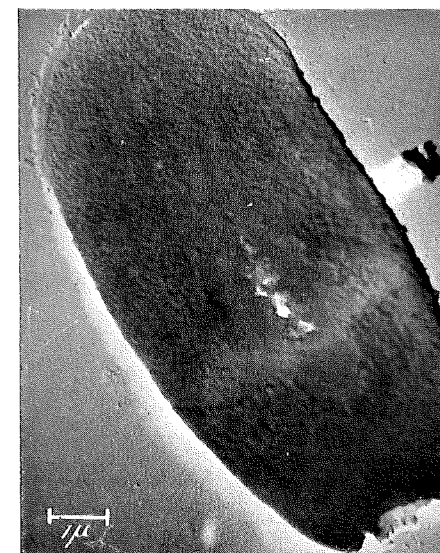

Fig. 1. Surface layers of head exposed, after ultrasonic treatment. Fibrillar cuff visible at base of head.

The posterior half of the head is covered by the post-nuclear cap which ends in a serrated edge. The cap is made up of coarse fibrous structure. The nuclear ring, mentioned previously, lies partially underneath the serrated edge and has often been confused with it. A ring or band at the base of the head, observed by light microscopists is simply the rim of the posterior boundary of the cap.

The acrosome cap consists of two parts: one, a short thick cap at the tip of the head, the other covering the whole anterior half of the head in a close fit.

Ultimately, there is a smooth membrane which may be the actual surface boundary of the nucleus. Penetrating still further into the head, a dense arc-like thickening is found near the anterior edge.

Ultrasonics detected structural weaknesses, and produced a puncture whose position proved to be constant. More extensive treatment eventually extended the damage in a series of gashes and small holes along the long head axis.

A cuff at the base of the head consists of sets of double fibrils, 200—250 mμ long and 45 mμ wide. They evidently play a part in the junction between head and neck. Clear-cut breaks at the neck are easily obtained, the cuff adhering to the head.

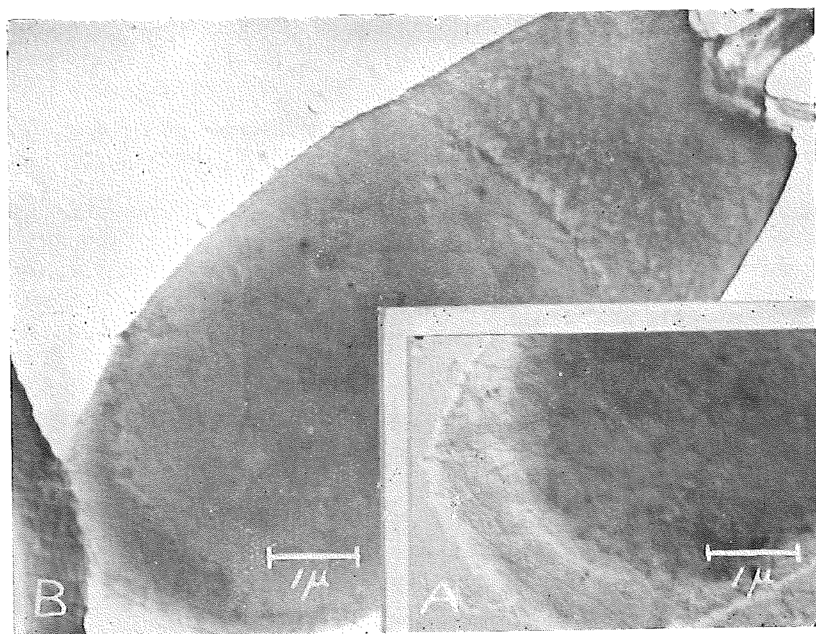

Fig. 2A. Head, showing fibrils in Galea Capitis.
B. Head, showing fibrils in anterior half. Fibril bundles with their surrounding coils visible in the neck region.

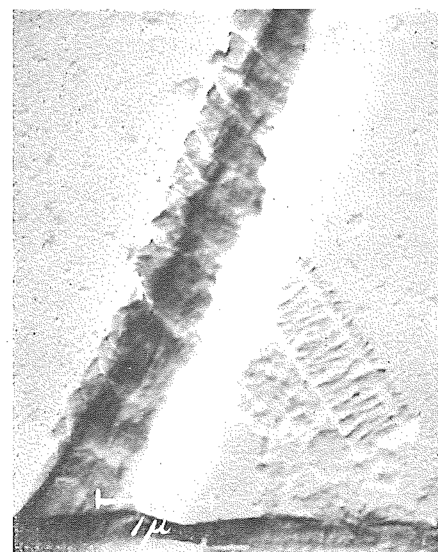

Fig. 3. Middle-piece band showing cross-striations, after treatment with ultrasonics.

Neck.

A well-defined spacing separates the head from the neck. The actual attachment appears to take place near the periphery, and is of a fibrous nature. Three fibril bundles are first observed in the neck, the middle one starting from a bulky central structure corresponding to the position of the centriole. Each bundle is surrounded by a coil. Constituent fibrils are often exposed but become more difficult to identify, 2μ or more beyond the neck junction. Each fibril is surrounded by its own coil which is genuine, and not simply a remnant of additional outer coils wound round the neck.

Middle-Piece.

With the beginning of the mitochondrial sheath the width broadens from 650 mμ to 800 mμ. The sheath itself consists of two bands which follow each other, inclined at 55° to the long axis. Half-turns of the band are 850 mμ long, and 250—300 mμ wide. Certain cross-striations on it have been a constant feature, and make an angle of 105° with the band axis. 6—10 are found in one half-turn. The width of these striations, while difficult to measure is ∽ 50 mμ and a doublet structure indicated more than once.

An outer membranous sleeve closely follows the band-coil of the middle-piece, while the coil itself is superimposed on a thick spiral wound round the axial filament core.

Principal Tail Part.

This is ∽ 500 mμ wide, with an outer doublet coil, inclined at 85° to the axis. Individual coils are 30 mμ thick. The axial filament runs down the entire length of the tail, encased in a membrane, and finally emerges to form the tail-end. Here a doublet coil can be observed, 10—15 mμ thick, and inclined at 75° to the axis. The tail-end is up to 3μ long, and 150—200 mμ wide. While flexible, close packing of its constituent fibrils gives it a circular cross-section.

Constitution of the Axial Filament.

It seems that one fibril bundle, presumably the central one remains intact throughout the main length of the tail, encased in a membrane. On the assumption that each bundle represents a triplet, the remaining six fibrils are loosely arranged round the solid bundle which consists of three pairs of fibrils. This brings the total number of fibrils to 12. This agrees with the numbers and widths of fibrils found in "brush-effects" (display of fibrils at breaks in the

tail), where 9 or 12 fibrils are observed, and thick fibrils can frequently be resolved as doublets.

The structure of the actual fibril itself is by no means simple. Excessive edge scattering and metal aggregation may sometimes simulate fine doublets and coils, respectively. There are definite indications, however, that a fibril is made up of two very fine filaments, lying side by side. It is surrounded by a coil, 10—15 mμ wide, possibly a doublet itself. Spacing of this coil ranges from 17.5—25 mμ, due to the fact that the fibrils are very easily extended which would alter the natural pitch drastically. They are capable of withstanding very great tension. The width of the fibril decreases with increasing distance from the neck. Measured towards the tail end, it is $\backsim$ 25—30 mμ. The constituent filaments are 5—10 mμ in diameter.

Membranes.

There are numerous membranes in the middle-piece and tail-systems. One surrounds the axial filament, while another is in close contact with the inside of the doublet helix of the principal tail part. The middle-piece has two additional membranes outside the mitochondrial sheath, and the whole sperm appears to be surrounded by a "lipoid capsule". This, however, is generally missing by the time the specimen is ready for electron microscope examination.

AN ELECTRON MICROSCOPE STUDY OF SENILE PURPURA.

R. REED, R. N. TATTERSALL and A. MILLARD,
Medical School and Dept. of Biomolecular Structure, University of Leeds.

Senile purpura is the name given to a special type of localised intracutaneous haemorrhage which only occurs in elderly people. It is rarely seen before the sixth decade, but by the ninth the incidence has risen to 25 %. The haemorrhages are found on certain parts of the body only, the extensor surface of the forearm and hand, and occasionally on the face. With increasing age these 'exposed' areas of skin are the site of degenerative tissue changes characterised by thinning, pigmentation and loss of elasticity of the skin, and in senile purpura these changes are particularly marked. Clinical studies clearly rule out any evidence of systemic disease, or of dietary deficiency in the aetiology of this condition. Histological studies of the skin by Unna and by Ejiri have shown that in exposed areas of skin there is a general increase in the elastic-staining fibres of the cutis at the expense of the collagen. These changes are not seen in the unexposed skin of the trunks and legs. Unna considered that the histological appearances were produced by a degeneration of both elastin and collagen to form 'collacin', whilst Ejiri concluded that the collagen fibres fragmented and disappeared, being replaced by an overgrowth of abnormal elastic fibres.

Specimens of skin were taken from the extensor surface of the forearm or the face as samples of *exposed* skin, and, for comparison, from the flexor surface of the forearm and trunk. Each specimen was divided in two, one portion being fixed in formol-saline and then stained with Lawson's elastic stain or acid orcein, the other being prepared for the electron microscope by removing the epidermis and subcutaneous tissue by dissection, then preserving in normal saline by refrigeration.

Examination of the orthodox histological specimens showed the following changes:

a. In s.p., the exposed (purpuric) skin showed a great increase in the elastic-staining fibres, with a diminution in the collagen. The fibres were thickened and fragmented, staining less deeply than normal elastic tissue.

b. In s.p., the unexposed skin showed no abnormality and the relative proportions of elastic and collagen fibres were the same as in control cases in the same age group.

c. In exposed skin from non-purpuric patients no gross abnormality was found, but it was noted that with increasing age there was some thickening and shortening of the elastic fibres.

d. Unexposed skin from non-purpuric patients showed no changes.

In the electron microscope examination of *unexposed s.p. skin,* only two types of fibres, namely collagen and elastin, were encountered. The former predominated and were easily recognised by their cross-striated appearance. * Moreover these fibres appeared of uniform width, about 1000 A.U., suggesting a proto-fibrillar unit. Elastic fibres were found only very occasionally, appearing as non-striated fibrous strands embedded in an apparently amorphous matrix. In general this skin had a very firm texture and gave very clean fibrils with little associated amorphous material.

Exposed s.p. skin, however, was much looser in texture, being torn apart very easily with dissecting needles. Electron micrographs showed the following types of components:

1. Non-striated fibres embedded in masses of 'amorphous' material (see Fig. 1).
2. Degenerate collagen fibres, degenerate in that they were short and inclined to be bent, in contradistinction to the fibres from unexposed s.p. skin, which followed very long and straight paths. These degenerate fibres were also associated with much 'amorphous' material (see Fig. 2).
3. Apparently normal collagen fibres, characteristically cross-striated. This material was present only in small amount, the commonest being non-striated fibres and degenerate collagen fibres associated with much 'amorphous' matter.

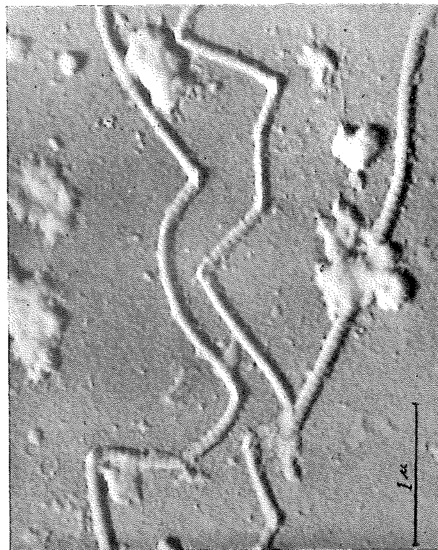

Fig. 2. Misshapen collagen fibrils from exposed forearm skin of a senile purpura patient.

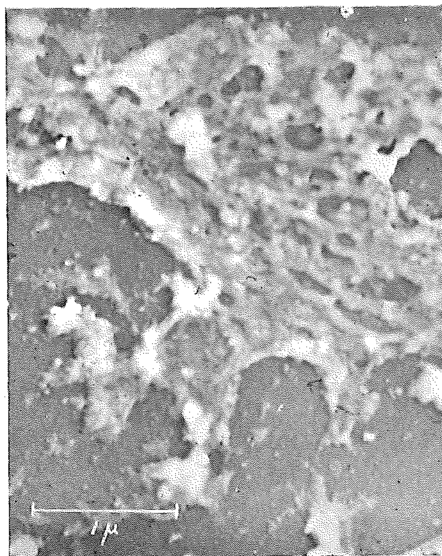

Fig. 1. Fibre network with amorphous material from exposed forearm skin of a senile purpura patient.

* See Fig. 1 of Prof. Astbury's introductory lecture on "Applications".

Exposed and unexposed skin from non-purpuric patients was similar in appearance to unexposed s.p. skin.

The main conclusion, therefore, from the work carried out so far is that exposed skin in senile purpura is characterised by the appearance of *a* collagen fibres that have become shortened and misshapen, *b* loose networks of non-striated fibres, and *c* much 'amorphous' material. The exact nature of the last-named, in particular, requires much further investigation.

Preliminary X-ray diffraction studies carried out in these laboratories by Prof. W. T. Astbury and Dr L. C. Spark are as yet inconclusive, but support the electron microscope observations in that some specimens give a better collagen diagram for the unexposed than for the exposed s.p. skin.

It has already been mentioned that the cutis of exposed s.p. skin stains as if it were mainly elastin. This does not, however, prove that the condition is associated with an actual transformation of genuine collagen into genuine elastin, for such a supposition seems very unlikely indeed. With the object of throwing further light on the matter, studies of elastic tissue also were undertaken, the structures examined being the media of the human aortic arch and tissue from a case of Ehlers-Danlos syndrome ('rubber skin'). Both these gave mainly elastic fibres with occasional collagen fibres, the predominant picture being one of fibres of various sizes embedded in an amorphous matrix. In the case of the Ehlers-Danlos tissue, some of the smallest fibres, which appear very uniform in diameter, can be seen twisted together to form thicker fibres (Fig. 3). This observation ap-

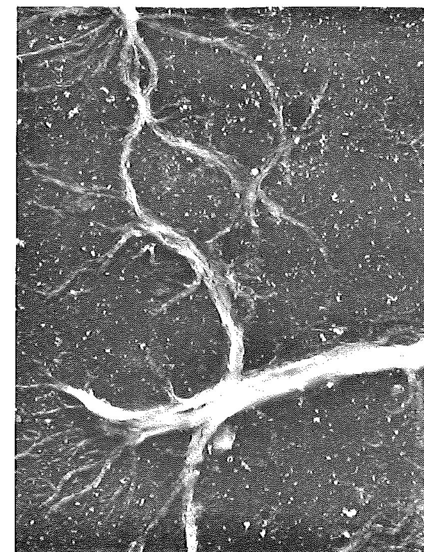

Fig. 3. Skin fibres from Ehlers-Danlos syndrome. ("Rubber-skin").

pears to conform with the results of Gross published during the course of the present work, and our characterisation of elastic fibres as non-striated and embedded in a matrix of smaller fibrils and amorphous material is in agreement with the earlier work of Wolpers.

As yet we are hardly justified in identifying the non-striated fibres found in exposed s.p. skin with true elastin. Exposed s.p. skin may indeed contain more elastic fibres than normal unexposed s.p. skin or skin from non-purpuric patients; but the presence of degenerate collagen fibres and much breakdown material would suggest rather that the abnormal condition is characterised, not by a transformation of collagen into elastin, but by a breaking-down of the former. Possibly, new elastic fibres appear at the same time, for it is a common observation that senile skin, while lacking in 'elasticity', nevertheless reveals on orthodox histological examination an increase in elastic staining.

The investigation is continuing and it is hoped that further developments will be reported on elsewhere.

STRUCTURE OF SEED MUCILAGES.

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and E.T.H. Zürich, Switzerland.

Mucilages are secreted by the hairs of many plants, but may be obtained in quantity only from certain seeds, such as cress, flax, quince, *etc.* These on soaking with water give a more or less viscous liquid. Other so-called mucilages are obtained from the medulla of *Astragalus* shrubs (Tragacanth) or from the tubers of *Orchidaceae* (Salep mucilages), but these are chemically of an entirely different nature from the seed mucilages.

Few of the seed mucilages have been thoroughly investigated chemically as yet, and generalization is at this stage impossible. They have been found to be of a polyuronide nature and galactose-glucuronic acids have been isolated from several of them (Norman [1]).

A large number of seed mucilages also contain cellulose. In view of modern knowledge of the structure of cellulose, it is extremely interesting to note that a form of cellulose exists, which in combination with acidic residues is soluble in cold water. As far as it was possible to generalize from the few samples investigated, Renfrew and Cretcher [2] assumed that the cellulose is chemically combined with the polyuronides to give a water-soluble complex.

The mucilaginous properties are no doubt associated with the polyuronides and not with the presence of dispersed cellulose.

We have studied these questions with the electron microscope and the results with different mucilages are very similar to each other.

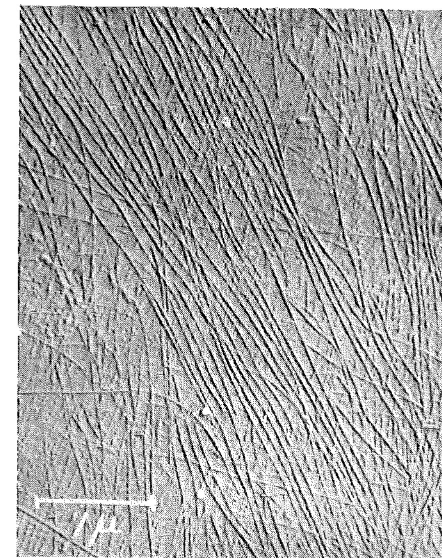

Fig. 1. Cress seed mucilage.

* Special Fellow of the Nat. Inst. of Health

For the preparation we used the following method: The seeds were swollen in cold water for two hours and then the mucilage rubbed off directly onto the collodion screen. The dried specimens were shadowed either with chromium or palladium.

In a previous study we found that the cellulose in cell walls is always crystallized in the form of uniform strands with a diameter of about 250 A.U. (Mühlethaler [3]). The cellulose membranes built by *Bacterium xylinum* showed the same form of microfibrils (Mühlethaler [4]). In Fig. 1 it is seen that in cress seed mucilages *(Lepidium sativum)* the cellulose is similarly developed. The surrounding substrate is completely amorphous. Presumably this complex can be water soluble because the network of the submicroscopic cellulose-fibrils is very extensible, and the surrounding heavily hydrated polyuronide chains are holding the fibrils in a highly dispersed state. If the polyuronic substance is hydrolytically removed by refluxing ten hours with 2.5 % sulfuric acid a visible separation occurs and a bulky residue remains. This residue is no longer water soluble. We found exactly the same structures in the mucilages of Quince *(Cydonia vulgaris)*, *Cobaea scandens* and Tragacanth. A different structure was found only in linseed mucilage. Here the cellulose is dispersed in the form of small particles (Fig. 2). They show no uniformity in thickness or length and it can often be seen that an end is splitting up into two or more smaller threads. The smallest have diameters of about 60—80 A.U. We must expect that this is the first stage of building up the long cellulose strands. If the isolating substrate is removed these particles have a tendency to form short fibers.

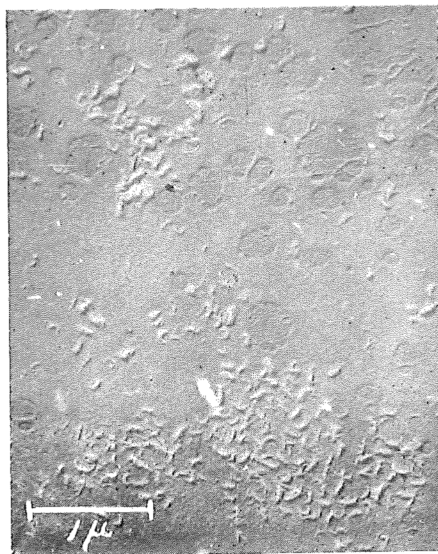

Fig. 2. Flax seed mucilage.

Our pictures are incompatible with earlier opinions that mucilage contains cellulose combined in some way with polyuronic acids.

These investigations were carried out in the Laboratory of Dr Ralph W. G. Wyckoff, whom I wish to thank for many helpful discussions.

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3. K. Mühlethaler, Biochim. Biophys. Acta 3 (1949) 15.
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Discussion.

Dr Houwink: From which cells and from which part of those cells does the mucilage originate and what happens to the cuticle?

Answer: The mucilage is produced in the epidermis cells of the seeds. Instead of a normal secondary wall a thick layer of mucilage is deposited. Usually the thickest lamella is on the outer wall. If the seed is moistened, the mucilage swells and the cuticle bursts.

STRUCTURE DES FIBRES NERVEUSES AU MICROSCOPE ELECTRONIQUE.

P. LÉPINE, P. ATANASIU et O. CROISSANT, Inst. Pasteur, Paris.

Les examens ont porté sur des fibres nerveuses isolées des nerfs périphériques chez le lapin, la souris et la grenouille. Ils ont été pratiqués en transmission directe ou après métallisation.

On observe des fibres ayant une structure périodique rapellant dans ses grandes lignes celle du tissu conjonctif. A la périphérie du nerf, les enveloppes sont indubitablement de structure connective mais à l'intérieur du nerf les structures analogues se retrouvent suggérant que la fibre nerveuse se décompose elle-même en faisceaux à structure périodique.

La section de troncs nerveux donne naissance sur les préparations à des images rappelant les classiques figures myéliniques.

Les examens ont été pratiqués sur du tissu nerveux non fixé et non coloré, observés immédiatement après dissociation mécanique des fibres au micromanipulateur.

De nouveaux examens après divers traitements sont nécessaires pour préciser la structure intime des éléments neuronaux.

Discussion.

Miss Friedlaender: What is the size of globules along single fibrils?

Answer by Dr Lépine: The size is about 55 mμ in diameter, with a narrow variation around the average. We have no explanation to propose as to what they are made of.

STUDIES OF HAEMOGLOBIN MOLECULES WITH THE ELECTRON MICROSCOPE.

R. S. M. REVELL and A. W. AGAR, Metropolitan Vickers Electrical Company, Research Dept., and J. O'H. TOBIN, Manchester Univ., Bact. Dept.

The electron microscope has been used with success by Wyckoff [1] in demonstrating the crystalline structure of plant viruses, his micrographs showing large numbers of viruses in regular array. Our work has extended such studies to haemoglobin, which is of a smaller size order, and which has already been studied by Perutz [2] by X-ray diffraction methods.

Our earlier experiments were made with specimens of haemoglobin from humans and horses, and our micrographs did indeed show individual molecules from these specimens. They do not easily crystallise, however, and the best results were obtained from rat haemoglobin, which is readily crystallised from aqueous solution.

Separation of Haemoglobin Sample.

The preliminary separation of the haemoglobin was similar in each case. The blood cells were washed six times with water, lysed, and the mixture was spun in a horizontal centrifuge for five minutes at 2000 r.p.m. This process removed the cell ghosts and left a supernatant containing the haemoglobin. This was filtered through a gradacol membrane of average pore diameter 0.83 μ and then spun in an angle centrifuge at 10,000 r.p.m. for an hour. The resulting liquid was used as the basis for the electron microscope specimens.

Preparation Techniques.

The specimens of human haemoglobin first used were prepared by drying down a drop of the specimen on to a grid and shadowcasting in the normal way. This preparation technique was successful in giving a number of individual molecules in the specimen, but there was a good deal of aggregation. The specimens of horse haemoglobin were accordingly studied by a preshadowed replica technique. This appeared to reduce the amount of aggregation but only very small areas of incipient crystallisation were found.

Rat haemoglobin was used as the basis for more extensive experiments. It was studied by means of the preshadowed replica technique, and also by drying down a drop of the solution on to a grid. In all cases the shadowcasting metal was a 40 % palladium gold alloy, and the films were of formvar.

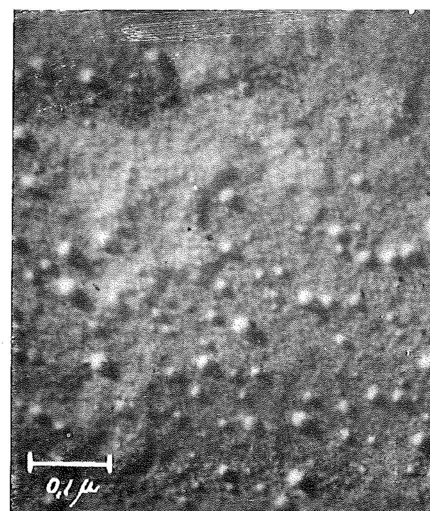

Fig. 1. Human haemoglobin molecules.

Results.

A typical micrograph of human haemoglobin is shown in Fig. 1. The smallest particles in the field which can be seen to cast shadows are individual haemoglobin molecules. They can clearly be distinguished from the background structure which is of the order 20—30 A.U. in size. Although the molecules have aggregated a good deal, the individual constituents of the aggregates can be distinguished in some places. There is some evidence of crystallisation at the top of the micrograph.

The study of horse haemoglobin yielded similar results, except that the aggregation was less pronounced. Some evidence of crystallisation was again apparent, though no large arrays were obtained.

The experiments on rat haemoglobin have proved rather more promising, and several different techniques were applied to this study. It was found that the crystals obtained were frequently too thick to allow penetration by the 50 kV. electron beam, and consequently only rough surface structure was observable. Drops of a warm solution were therefore dried down on to the grids, so that the haemoglobin crystallised out as the water cooled and evaporated. Crystals of rather smaller size were obtained by this method, and they did show regular molecular arrangement. However, such arrays were now too small to yield useful quantitative measurements on the molecule size and orientation, and preshadowed replica technique was adopted as being more useful.

Results have so far been restricted because it has been found that the crystals generally adhere to the replica film, so that those of a size likely to yield useful quantitative information are generally too thick to allow electron penetration. An example of this is shown in Fig. 2. Some structure is visible on the surface of the crystal, but it cannot be resolved into molecular arrays. Some small

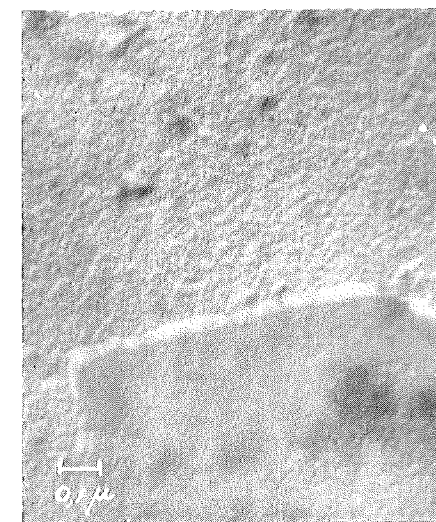

Fig. 2. Rat haemoglobin molecules.

molecular arrays can be discerned in the background.

Preliminary measurements from the two micrographs shown give an approximate value of 80 x 60 x 60 A.U. size. This can be compared with Perutz's results which show the molecules to be cylinders 34 A.U. in height and 57 A.U. in diameter. Our measurements are complicated by the uncertain knowledge of metal thickness deposited on the specimen (believed to be of the order of 20 A.U. in the direction of shadowing). More accurate measurements can be expected when larger crystal arrays are available.

It is considered that these results roughly confirm the X-ray diffraction measurements of the size of the haemoglobin molecules. It is expected that refinements in the preparation techniques will yield more accurate results, and perhaps give some clue as to the way in which the crystal structure is built up.

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1. Wyckoff *et al.* Science, 102 (1945) 277 etc.
2. Perutz, Proc. Roy. Soc., 195 (1949) 474.

Discussion.

Dr Reed: Has the appearence of blank films shadowed at the same time as the loaded films been studied? When dealing with such small particles it is extremely difficult to distinguish between actual particles and the grain of the background.

Answer: This has been done, and there was no danger of confusion between the molecules and the metal grain. The metallic grains were of the order of 20 A.U. in diameter, that is, four times smaller than the molecules, and the molecules could also been seen to cast distinct shadows. The features of the substrate could be of a larger size order in the case of the film mounts, but we have obtained exactly similar results from the preshadowed replica technique where glass is the substrate and detail is negligible.

SOME OBSERVATIONS ON BACTERIA GROWING ON SURFACES.

A. L. HOUWINK, T.P.D., E.M.Div., Delft.

The study of the bacteria of the soil induced scientists to look for a method by which the organisms could be observed directly, that is: without preliminary culturing in some nutrient medium.

This problem would not be hard to solve if only it were feasible to identify and to count the bacteria which live on the surface of the soil particles, and in the water between these particles, by simply putting a bit of soil under the microscope.

This, however, is impossible. Still a method has been devised which enables us to get at least a faint idea of the bacterial flora on the surface of these particles. The Russian scientist Cholodny [1] deposited ordinary microscope-slides in the soil. After some days or weeks these were dug out and then they were found to be covered with a large number of organisms which had attached themselves to the glass, so as not to be washed away by rinsing the slide. Such a preparation can be stained and examined with the microscope.

It is self-evident that Cholodny's technique was really more apt for the study of the microflora and fauna of water than for the use it had been devised for. Indeed good preparations may be obtained by simply hanging a slide in water. This has been put in practice by Henrici [2], ZoBell and Allen [3] and others.

A collodion membrane may be used just as well as a slide. Collodion membranes will float on water or on a nutrient medium for many days without being damaged. When the experiment was made with water of one of the Delft canals it was found that after a couple of days a number of bacteria had settled on the collodion. The majority of these could not be identified, because most species of bacteria are morphologically similar. Some, however, we readily recognised to be a *Caulobacter*-species.

This bacterium we had already in a pure culture. Actually it has been isolated from a bottle with distilled water. This bottle had been cleaned with tap water. It has only seldom been observed by microbiologists as it does not multiply as fast as the other species, and therefore is never found in the usual enrichment cultures. Up till now we did not find a selective medium promoting its growth and in the meantime checking the development of other organisms.

The specimen which is shown in Fig. 1 is just about to divide by fission. The upper cell will swim

Fig. 1. *Caulobacter* spec., sessile. Stalk attached to collodion membrane.

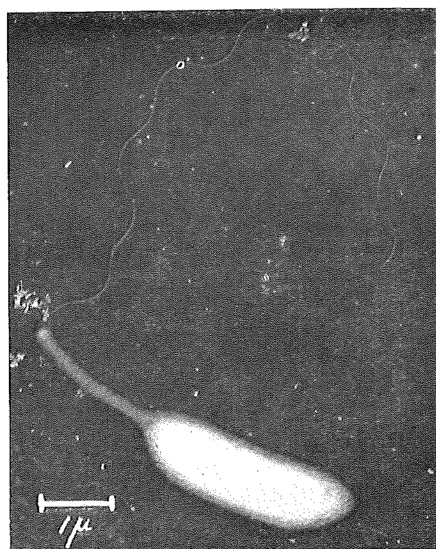

Fig. 2. Same as Fig. 1. Long flagellum at the base of the stalk.

away by means of a slender flagellum, but after a while it settles on some surface. I suppose that it always attaches itself at the flagellated end since many young cells with a more or less well developed stalk have a flagellum at the base of the stalk, but none have a flagellum at the free end. So it was concluded that the flagellum which is shown in Fig. 2 is the old flagellum which is no longer used as a propelling organ. When the cell is about to divide, however, it grows a new flagellum at the free end.

A *Caulobacter* species was first found some 40 years ago by Miss Jones [4] at Chicago. She noticed that it tends to form rosettes. The same property is displayed by the *Caulobacter* species which is represented here.

One of the advantages of the procedure of making the bacteria settle on a floating membrane is that the preparation can be rinsed with distilled water so that the organisms are clean. This was one reason to investigate if other, well known species also will attach themselves. The sitting habitus appears not to be a rare quality.

Escherichia coli, the ordinary coli bacillus, for instance, can attach itself to collodion. There seems to be a relation between the concentration of nutrient substances on the one hand and the number of attached bacteria on the other, namely the lesser the concentration the higher the percentage of attached cells.

In Fig. 3 numerous fine threads are seen to radiate from the bacterial surface in addition to flagella. In this preparation the flagella appear to have been dragged away by the retiring water during the drying process but these filaments radiate in all directions thus enabling us to conclude that they were attached to the membrane. One might call them surface growing filaments. The same

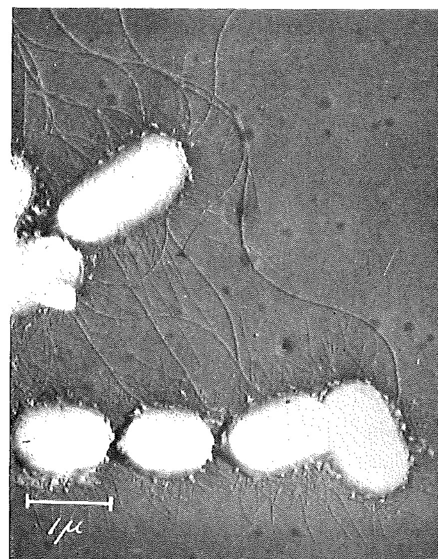

Fig. 3. *Escherichia coli*, attached to collodion membrane. Flagella and "surface-growing filaments".

sort of things we found in *Pseudomonas pyocyanea.* These bacteria form filaments when they are grown on agar. Unlike *E. coli, Ps. pyocyanea* makes them only at the ends of the cell. The fact that these filaments are peritrichous in *E. coli* whereas they are polar in *Ps. pyocyanea* seems to be purely incidental. We did not find filaments on free living individuals of the same species. It might be that in this case the material is broken off in little pieces by the movements of the bacterium, but there is nothing to prove this.

These filamentous structures have been observed already by several electron microscopists. Still it is not known if they have any function. It should be mentioned here that some forty-five years ago the German bacteriologist Hinterberger [5] found similar fine threads in stained preparations of several species. He called these structures "Myzel" and suggested that they will serve the uptake of nutrient substances.

Perhaps the appearance of creeping filaments on the surface of a nutrient medium did remind those, who study tissue cultures, of the pseudopodia of cells which develop in similar circumstances. By no means I want to suggest that surface growing filaments and pseudopodia are homologous structures. Up till now we can not even be certain that the bacterial filaments are parts of the living protoplasm. Still in both cases the presence of a surface seems to have a marked influence on the morphology of the organism.

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ON MULTIPLE MEMBRANE STRUCTURES IN THE RETINAL RODS OF THE GUINEA PIG EYE AND IN NERVE TISSUE.

FRITIOF SJÖSTRAND, Department of Biology, M.I.T., Cambridge, Mass.

This paper will deal with highly specialized biological structures, namely the type of sensory cells in the eye that are called the retinal rods, and, furthermore, the nerve cells. The aim of this study was to analyze the structural background of irritability, which in these cell types is especially well developed. I shall here talk mostly of the various techniques used, as this might be of a more general interest.

As long as we lack suitable sectioning techniques that make it possible to prepare by routine sections of less than 500 A.U. in thickness, we have to try *fragmentation techniques* on biological material for studying the finer structures.

When fragmenting the material, we destroy the arrangement of the structural elements and run into difficulties regarding the localization of different structures within the cell. Therefore, we have to start with an extremely uniform material representing as small and as uniformely structured a part of a cell as possible.

In the case of the retinal rods which constitute slender cell elements, oriented parallel to each other and perpendicular to the surface of the retina, there is one part that is of primary importance when studying this problem, viz, the part characterized as the *outer segment*. This segment represents the part of the cell that is responsible for the extremely high irritability of these cells with regard to light.

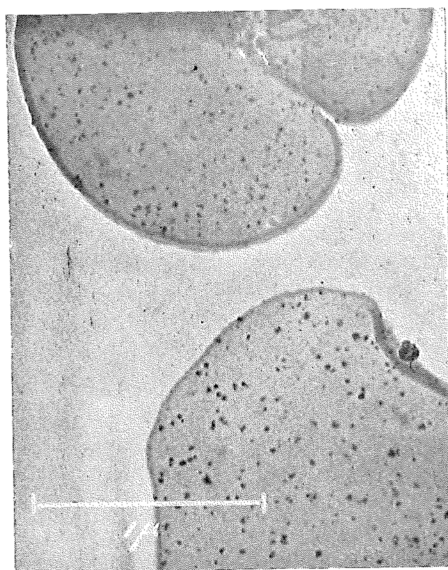

Fig. 1. Dispersion of fragmented rod outer segments with discs of different thicknesses. The upper disc is a unit disc showing the typical arrangement with very dense spots scattered over the disc surface.

In order to start from a uniform material the guinea-pig eye, which contains only rods and no cones, was selected, and the outer segments were broken off from the rest of the retina. In this way, a pure dispersion of outer segments was obtained.

The material was fixed in 2 % osmic acid which, according to W. J. Schmidt, does not destroy the form birefringence due to the orientation of protein structures in the rod outer segments. Then the dispersion of rod outer segments was subjected to sonic fragmentation, using a frequency of 9 Kc.

This resulted in the cleavage of the cylindrical rod outer segments into very thin discs, whose diameters equalled about 2 μ *i.e.* similar to the diameter of the rod outer segments (Fig. 1). The fragmentation can be followed through its intermediate stages, showing a successive breaking up of the outer segments.

The varying thickness of the discs was measured on shadowed preparations, using chromium and an angle of incidence of 10°. Fig. 2 demonstrates a characteristic distribution of thicknesses, the thinnest discs being 70—80 A.U. in thickness and the thicker discs representing thicknesses corresponding to multiples of pairs of these thinnest discs. At continued fragmentation, the thinnest discs were observed to break up into fragments of no characteristic form. They are, therefore, described as the unit structure of the rod outer segment. The thicker discs represent piles of pairs of these unit discs.

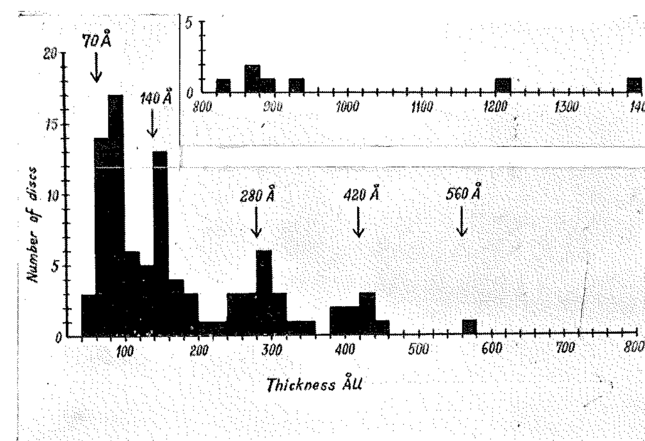

Fig. 2.

The unit discs have a fairly complicated structure with an outer edge of a breadth of 100—200 A.U., showing higher electron scattering power than the rest of the discs with the exception of very dense spots with a diameter of 50—300 A.U. which are fairly uniformly distributed over the surface. It is impossible to decide from the unshadowed electron micrographs whether the differences in contrast depend upon differences in mass density or in thickness. It may be inferred from

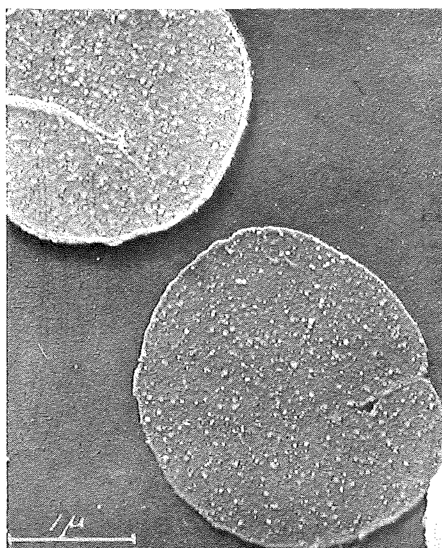

Fig. 3. One unit disc from rod outer segment (the lower disc) and one quadruple disc. Shadowed with chromium, angle 10°.

the shadowed pictures (Fig. 3) that the higher electron scattering power of the outer edge may be due to the fact that this edge represents a thicker region of disc. The disc membrane is accordingly only about 30 A.U. in thickness (Fig. 4).

The dense spots also correspond to thicker parts, but the height of these knobs over the disc membrane is only about 70 A.U., so that the greater thickness alone cannot be responsible for the great scattering power of these parts. It seems possible that there may exist some difference in mass density through either a crystalline arrangement, or an accumulation of heavier atoms. Osmium is not responsible for this marked contrast, the same contrast occurring also in preparations fixed in formalin.

Almost regularly an incision is to be seen towards the centre of the disc at one point of the circumference. The unit discs easily rupture at this point and may then form circle-sectors. The entire preparation has to be performed in the cold at about + 4° C in order to preserve the discs better.

The data available regarding these discs facilitate a discussion of their arrangement in the rod outer segment. Judging from the distribution of thicknesses the unit discs have a characteristic tendency to stick together in pairs. When preparing in room temperature, the thinnest elements that could be isolated represented pairs of unit discs. But when preparing in the cold the unit discs were isolated. Apparently, the forces acting between the unit discs in a pair are stronger than the forces acting between the disc pairs.

The unit discs, therefore do not seem to be uniformly piled up.

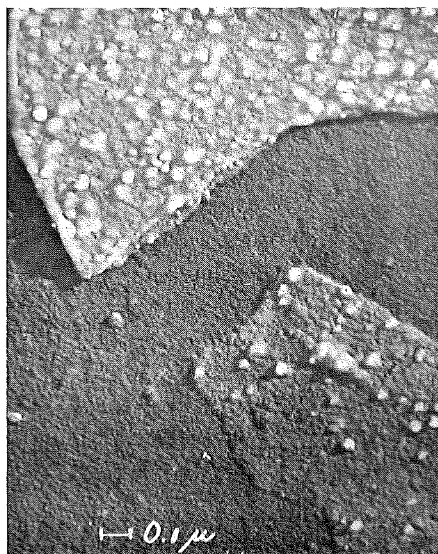

Fig. 4. Fragment of the disc membrane, shadowed with chromium, angle 10°, showing the minute thickness of this membrane and the granular structure of the disc knobs. In the upper part of the picture a doublet with a rupture of the disc membrane.

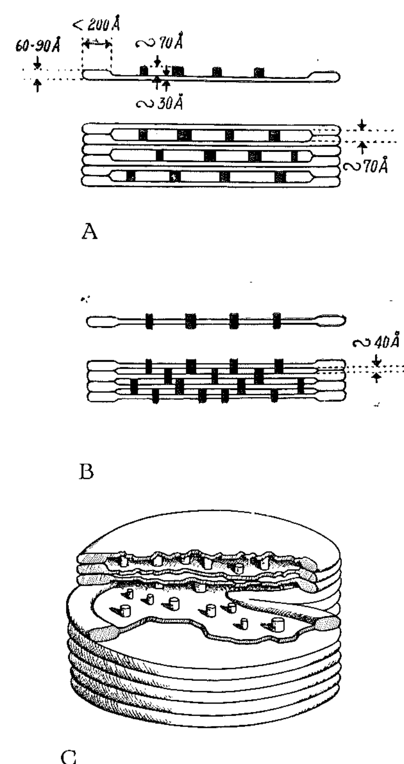

Fig. 5. The different possibilities for the arrangement of the unit discs in the rod outer segment of the guinea-pig retinal rod.

A. Unit discs arranged in pairs.

B. Unit discs arranged with uniform spacing throughout the segment.

C. Perspective scheme of the arrangement of the unit discs with an arrangement corresponding to alternative A.

The knobs on the disc membrane rise to about 70 A.U. over the surface of this membrane. This measure probably indicates the distance between two adjacent unit discs. Supposing this to be so, we shall arrive at an arrangement of the discs corresponding to scheme A in Fig. 5. In this case the forces acting between the discs constituting a pair may very well be different from the forces acting between the pairs. The uniform piling up of the discs (scheme B, Fig. 5) does not conform to this.

We know from polarization optical data that lipid molecules are arranged longitudinally to the long axis of the outer segment, and that the protein constituents are oriented in planes perpendicular to this axis. W. J. Schmidt has presented one simple alternative which fits in with these data, assuming a structure with alternating protein and lipid layers.

With a distance of 70 A.U. between the unit discs in a pair corresponding to the height of the knobs, there would be sufficient space for one double layer of lipid molecules between these discs. According to data obtained by X-ray analysis on dried nerve lipids by Bear, Palmer and Schmitt (1941), the thickness of one double layer would be 64 A.U.

The observations made by electron microscopy fairly agree with what was expected from the polarization optical analysis. It is interesting to note that the conclusions that positive uniaxial form birefringence may be due to an orientation of rodlets have been verified in many cases, *e.g.*, in muscle and collagen. This study of the rod outer segments confirm by direct observation that a negative uniaxial form birefringence may depend upon the orientation ol platlets. The direct method of electron mircoscopy has thus beautifully established the basic conclusions derived from this indirect method. This is of the utmost importance since polarization optical analysis can often be performed on living material.

With the exception of some regularly appearing filamentous structures in these preparations, the whole outer segment seems to be composed of these unit discs. According to the

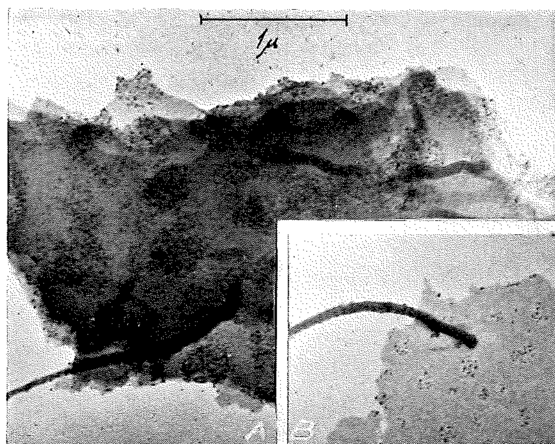

Fig. 6A and 6B. Membrane structures from nerve tissue presumably from the myelin sheats. 6A shows a pile of such membranes; 6B a single one.

data obtained, the outer segment apparently consists of about 2.000 unit discs.

In studying a structure like the rod outer segments, which is composed of such uniform structural units, the fragmentation technique is quite adequate. The fragmentation can easily be followed in intermediate stages and we well know the origin of the structures analysed. When trying to put the unit structure together more in detail we have to propound some hypotheses. Regarding the filamentous structures observed, it is difficult to ascertain their origin and arrangement.

The situation is even more complicated when proceeding from a more complex material, such as a peripheral nerve made up of connective tissue, Schwann cells, myelin sheaths and axis cylindres. By using a similar technique, it was possible to isolate thin membranes from guinea-pig nerves. These membranes were often collected in thick piles or single. (Fig. 6 A and B). The thickness of the membranes is about the same as that of the disc membranes of the retinal rods.

From polarization optical data we can conclude that there is a great resemblance between the structure of the myelin sheaths and the rod outer segments, and it is conceivable that the membranes, isolated from nerve tissue, derive from the myelin sheath, probably constituting the protein material of this structure.

We lack direct evidence of this, and we cannot build only on fragmentation techniques. We have to prepare thin sections to find out the arrangement of different structural elements.

But, even with an ideal sectioning technique at our disposal we shall find fragmentation techniques extremely useful, seeing that this constitutes an outstanding method for preparing the individual structural elements for a more detailed study.

I should like to point out that the most important question of biological electron microscopy today is the development of sectioning techniques, which make it possible to utilize the

resolving power of the electron microscope in the study of sections. The methods of preparing and conserving the biological material before sectioning or fragmentation furnish equally important problems.

Discussion.

Dr Walters: Have any observations been made on mammalian cones? These should be of importance in throwing light on the Stiles-Crawford effect (directional light sensitivity of human cones).

Answer: An analysis on retinal cones is planned.

Dr Kreger: Has any X-ray investigation been done on the rods in orde to find any crystal spacings that might yield more evidence concerning the structure and nature of the discs and their nods?

Answer: No X-ray data are available yet but such an analysis is planned.

Prof. Roelofsen: Are the knobs appearing on the slices real or may they have been caused by drying out of the rest of the material of the slices? If so could this be settled by making replica's of the slices before they are dried out?

Answer: The problem regarding the real nature of the knobs is not yet solved. They might very well represent artifacts due to fixation. This problem can be attacked by using freezing-drying fixation. At the moment the knobs may be used to indicate the distance between adjacent disc membranes but a detailed discussion regarding their structure is of no value until they are demonstrated as real vitally preformed structures.

Miss Friedlaender: Is any surface structure such as a dip visible in the thicker knobs between the plates?

Answer: There is a definite structure in the knobs. They are composed of aggregates of spherical bodies about 50 A.U. in diameter often arranged in a regular way resembling a crystalline arrangement.

STRUCTURE DE LA MEMBRANE DES SPORES DE MYXOMYCETES.

M. LOCQUIN, Laboratoire de Cryptogamie du Museum National d'Histoire Naturelle, Paris.

L'étude au microscope électronique des ornementations des spores des Myxomycètes montre qu'elles peuvent avoir deux origines: épisporique ou endosporique.

1. Ornementations épisporiques.

A. Type échinulé.

Trichamphora pezizoidea illustre de façon typique ce type d'ornementation. Son échinulation est à la limite de visibilité du microscope optique. Individuellement chaque ornement est une fine épine atténuée à son extrémité.

Leocarpus fragilis a des ornements moins nettement définis, la spore étant plus verruqueuse qu'échinulée.

Brefeldia maxima dont la spore à un grossissement faible montre une échinulation serrée, en apparence analogue à celle de *Tr. pezizoidea,* présente des ornements capités très particuliers qu'on ne retrouve parmi les autres espèces que nous avons pu étudier que chez certaines races de *Fuligo septica.* La partie terminale de chaque ornement est renflée en une tête ou plutôt un capitule de petites excroissances irrégulières et tronquées.

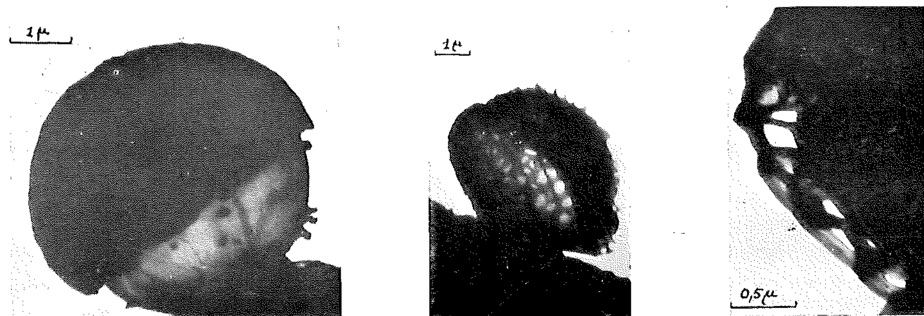

Fig. 1, 2 et 3. Spores de *Reticularia lycoperdon.*

B. Type réticulé.

Il est très fréquent et bien reconnaissable au microscope optique. Le microscope électronique ne révèle aucune particularité digne d'être notée.

2. Ornementations endosporiques.

Il est nécessaire d'avoir recours à une préparation particulière de l'objet pour la mettre en évidence. Une bactérie aérobie sporulée indéterminée, normalement associée dans la nature aux plasmodes de myxomycètes, a fourni un extrait capable de dégrader les composants de la paroi sporique. L'action ménagée de cet extrait sur les spores de *Reticularia Lycoperdon* permet dans bien des cas une séparation aisée de l'épispore et de l'endospore. Sur la Fig. 1, l'épispore a été détruite sur la moitié de sa surface; sur l'endospore ainsi mise à nu quelques ornements primitivement noyés dans l'épispore subsistent. En 2 l'épispore partiellement rétractée, à structure alvéolaire a dégagé les ornements endosporiques qui occupaient les alvéoles. A un plus fort grossissement en 3 on aperçoit la charpente du réseau épisporique nettement détachée de l'endospore.

REMARKS ON THE CRYSTALLINE STRUCTURE OF SOME PLANT VIRUSES.

V. E. COSSLETT, Cavendish Laboratory, Cambridge.

1. The crystals of plant viruses are of interest from two viewpoints. In themselves, for the light they throw on virus structure, and especially on the binding forces which hold particles in a regular lattice, with spacings of up to 500 A.U. Secondly, for crystallography generally, since they are the only lattices we can directly view, giving evidence as to how crystals are built up .

2. Methods which may be used in investigating them include:

a. *Optical,* giving the form of the micro-crystals, confirming their true crystallographic individuality.

b. *X-ray,* giving the lattice form in detail. Exposures are long (up to 100 hours), but the method can be used for wet as well as dry crystals.

c. *Electron Microscopy;*

i) Pseudo-replicas (Wyckoff).

ii) Direct shadowing, of crystals deposited on supporting film.

iii) Direct transmission pictures of thin crystalline fragments or platelets on collodion or beryllium films.

These methods are complementary, as illustrated by some experiences with two viruses: Turnip Yellow Mosaic (TYM) and Bean Stipple Streak (BSS).

3. Transmission pictures of *unshadowed* fragments of TYM crystals, dried down on to Beryllium (Cosslett and Markham [1]) or collodion (Fig. 1) films, exhibited near-hexagonal pattern of *holes,* the solid

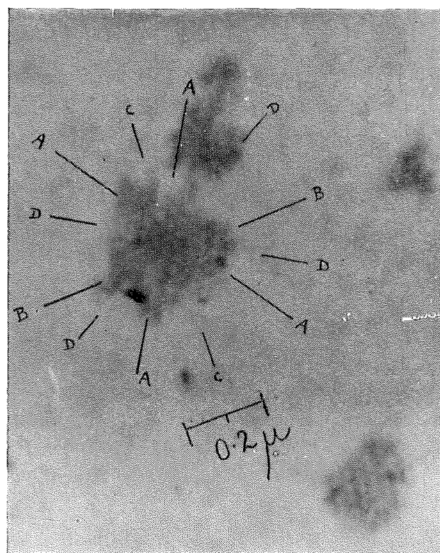

Fig. 1. Crystalline fragments of Turnip Yellow Mosaic Virus, on collodion supporting film. (transmission picture, unshadowed).

parts of the lattice showing less regular form. Measurement along the indicated directions confirmed that this is a view through a diamond lattice: a projection normal to the 110 face, along the diad axis. The particles then overlie each other leaving a set of clear holes through the lattice which are arranged in a hexagonal pattern that is non-regular, owing to the non-planar bonding.

The lattice spacing deduced from these pictures agrees well with X-ray results, both for the virus intact and after removal of nucleic acid; X-ray work also gives the dimensions in the wet state (Bernal and Carlisle [2]).

	Intact virus.	Less nucleic acid	
Dry crystals			
	195 A.U.	215 A.U.	— E.M.
	228 A.U.	238 A.U.	X-rays.
Wet crystals			
	306 A.U.	318 A.U.	X-rays.

Calculation shows that the shrinkage on drying corresponds to a water content of 85 %. The increase in size on extraction of nucleic acid would be doubtful on electron microscope evidence alone, but the X-ray confirmation is convincing. It is an interesting problem why the nucleic acid, which is essential to pathological activity, causes a compacting of the virus particle. The smaller dimensions given in each case by the electron microscope, compared with X-rays, is believed to be real, and probably due to the extra drying on insertion in the electron microscope.

Of the few viruses of known crystal structure, most are of cubic close-packed (face-centred) type or nearly so. The diamond structure of TYM argues strongly directed binding forces, tetrahedrally arranged, which sets an intriguing problem of their origin. Possibly the individual particles are truncated tetrahedra in form (Bernal and Carlisle), not spherical when in the lattice. Free particles show no departure from spherical form, but this is also the case with Southern Bean Mosaic, for which Wyckoff has nevertheless shown a definite axial ratio of 1 : 1.4 in the lattice. It may well be that free particles are gathered into spherical form by surface tension forces during drying. A better resolving power will be needed to test the true sphericity of these virus particles.

4. The Bean Stipple Streak virus was brought to Cambridge by Dr Van der Want of Wageningen, and purified by Dr R. Markham. It forms

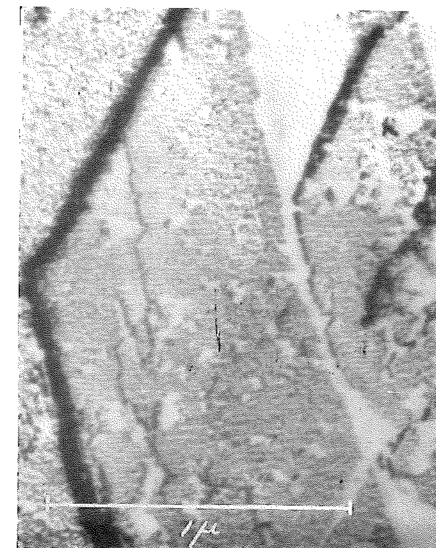

Fig. 2. Bean Stipple Streak Virus crystal. (pseudo-replica in gold).

well-defined flat platelets. Shadowed replicas show (Fig. 2) the lattice structure to be of close-packet form in the flat faces, but with a marked

distortion from true hexagonal form. One angle is nearly 60° but the others are 45° and 75°, approximately. The closest packed rows have a spacing of 150 A.U., the loosest of 210 A.U. The particles are certainly therefore elongated and most probably cylindrical in form. The exact structure cannot be determined until some of the side faces are made visible, which is difficult to achieve in this case, owing to the small thickness of the crystals. Such evidence as so far exists points to a distorted face-centred cubic lattice. X-ray evidence is not yet available, nor are there any data for the water content. The high degree of perfection which survives drying, however, argues that the shrinkage is not so great as for TYM. In general it may be supposed that the more a structure departs from close-packing, the higher will be its water content, and thus the greater the shrinkage on drying and the lower the probability of obtaining intact dry crystals.

The BSS virus is serologically very closely related to the Rothamsted Necrosis virus, for which Wyckoff [3] finds a monoclinic distortion of the face-centred cubic lattice. However, the close-packed faces of the latter are quite regular and there is no evidence of elongation of the particles, which are spheres of 140 A.U. diameter.

The BSS platelets are so thin that direct transmission pictures of crystals shadowed on a supporting film may be obtained. These naturally show the surface lattice less clearly, but also contain a number of long strips (Fig. 3) at the side of crystals. These are not found in replicas made by the Wyckoff method, possibly being destroyed when the collodion solution is flowed over the preparation. They do not show visible lattice structure, but may possibly be planes of particles detached from the side of crystals. The direct shadowing process may also have an advantage over the replica method of showing the crystal faces exactly in the form left by drying, unaffected by the exposure to liquids which the latter entails. This is important for

Fig. 3. Bean Stipple Streak Virus crystal. (shadowed with gold paladium, *in situ*).

the study of the way crystals are built up. The layer structure of the surfaces shows clearly how they are formed by the laying down of successive planes of particles, but with local dislocations. As Wyckoff has pointed out *(loc. cit.)*, the detailed study of such micrographs should provide valuable information for crystal physics. It would be a great advantage, however, to have a wet replica process, since one cannot be sure how much of the dislocation visible in a dried crystal may be a drying artefact.

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Discussion.

Prof. Astbury mentioned that other crystals (tropomyosin) contain even more water than TYM, and suggested that it was unnecessary to invoke long-range binding forces: the structure could be held together by polarisation of the intervening water molecules. This argument, however, begs the question as to how the polarisation is caused, and directed along in particular axes.

Dr McFarlane suggested that the smaller electron microscope values, compared with X-rays, might be due to electron bombardment rather than vacuum drying.

Mr Nixon pointed out the evidence for BSS being a tobacco necrosis virus, very similar to the Rothamsted strain. His electron micrographs showed no visible departure from spherical form, in isolated particles.

THE FLAGELLAR STRUCTURE OF SOME PROTISTA.

Y. T. CHEN, Botany School, Cambridge, England.

The structure of the flagella of eight species of flagellates, *Euglena viridis, Euglena gracilis, Lepocinclis spec., Trachelomonas volvocina, Trachelomonas zorensis, Phacus pleuronectes, Peranema trichophorum* and *Chilomonas paramoecium,* all maintained in culture, were studied with the electron microscope. The specimens were fixed either with 0.2 % osmic acid or 5 % formalin and washed in small tubes of distilled water, sometimes aided by centrifuging. The electron microscope used was that in the Cavendish Laboratory and was operated by Dr V. E. Cosslett, to whom I am deeply grateful for his valuable help.

The gross morphology of the flagellum varies in different species. In both species of *Euglena,* and most probably in *Phacus pleuronectes* too, the flagellum is ribbon-shaped (Fig. 1a). Sometimes this feature can also be demonstrated under the light microscope by Loeffler's stain. In all the other forms, the flagella seem to be cylindrical. The flagella of *Chilomonas paramoecium,* which have been described by some writers as ribbon-shaped, appear cylindrical in the electron micrographs.

It is generally believed (*e.g.* Kudo [1]) that the flagellum is wider at the base, tapering towards the end. This has not been confirmed. In all the species studied, the flagellum is of the same thickness throughout the entire length, except the part lying within the reservoir which is narrower owing to the absence of the outer sheath.

Differentiation into core and sheath is found in all the species studied. The core extends from the basal granule on the wall of the reservoir up to the tip of the flagellum. In *Euglena viridis, E. gracilis* and *Phacus pleuronectes,* the micrographs show many longitudinal striations on the core of the flagellum (Fig. 1), indicating a fibrillar structure. Dellinger [2], using a light microscope described 4 fibrils in the flagellum of *Euglena* sp. and of *Chilomonas paramoecium,* while Korschikoff [3] believed it consists of many more fibrils. By electron microscopy, Schmitt, Hall and Jakus [4] found 9—11 fibrils in the flagella of *Trichonympha,* while Brown [5] demonstrated only two thick fibrils in *Euglena gracilis* and *Astasia Klebsii.* The descrepancy in the number of fibrils may possibly be due to the finer fibrils collecting into bundles, a condition found in mammalian sperm tails (Bretschneider [6]). In *Euglena viridis, E. gracilis* and *Lepocinclis* spec., we found indications of many fine fibrils in the axial core. In *Trachelomonas volvocina,* how-

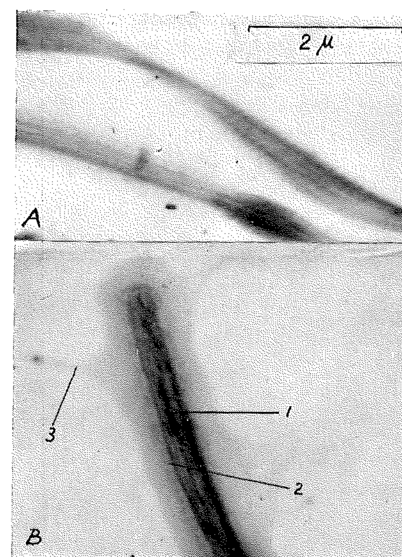

Fig. 1. Ribbon shaped flagella of *Euglena viridis.*
A. Note the longitudinal fibrils in the axis and the cross striations in the sheath.
B shows the axis *1,* the sheath *2,* and the mucilaginous layer *3.*
OsO_4. Centrifuged.

ever, we found two thick threads similar to what Brown observed in *Euglena* and *Astasia.* These are most probably two fascicles of fine fibrils.

The sheath covers the entire flagellum outside the reservoir, even up to its very tip. The root of the flagellum, when it is squeezed out from the reservoir, is seen to consist of core only. In most of the species studied, the sheath shows cross striations similar to those described by Brown. These are believed to be fibrils spirally arranged around the axial core (Fig. 1). In *Peranema trichophorum,* however, probably owing to their shortness, the fibrils radiate from the core giving the whole flagellum the appearance of a test tube brush.

Lash-like processes have been found on the surface of the flagellum (outside the sheath) of *Euglena viridis, E. gracilis* and *Lepocinclis* spec. (Fig. 2). But, instead of appearing as stiff independent hairs as under the light microscope (Vlk [7] *et al.*), they are, in electron micrographs, finer, softer, and sometimes matted together. They usually occur along one side only, but occasionally are seen on both sides of the flagellum. When present only on one side, it is always the convex side of the flagellum which bears them. The increase in thickness of these structures by light microscopical technique seems to be due to the effect of mordanting which, for example in Loeffler's technique using tannic acid and ferrous sulphate, gives a heavy precipitate around the specimen. Centrifuging has a considerable effect on these processes. In centrifuged specimens of *Euglena viridis* (Fig. 1), these processes ap-

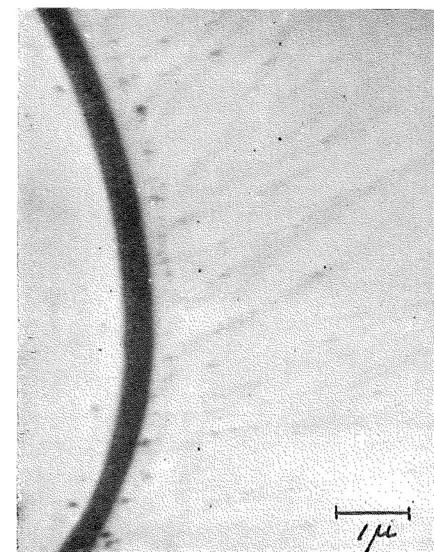

Fig. 2. Flagellum of *Euglena gracilis,* showing the lash-like processes along the convex side.
OsO_4. Not centrifuged.

pear merely as a mucilaginous layer surrounding the whole surface of the flagellum, which occasionally gives rise to a few pseudopodium-like processes. When the flagellum is much bent, this layer, like the processes in non-centrifuged specimens, is much thicker on the convex than on the concave side. In shadowed specimens, such thicker regions give the appearance of frills. It seems that these so-called lash-like processes, which are mucilaginous in character, are liable to change their form according to treatment. Although they are normally present all around the flagellum, since they are soft, they trail behind when the flagellum is beating, with the result that they are present only on the convex or trailing side. The single spiral row of these processes found in certain species by Vlk may also be explained in this way, as the flagellum usually beats in a spiral manner.

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ETUDE DE L'APPAREIL NUCLEAIRE DE QUELQUES BACTERIES EN MICROSCOPIE ELECTRONIQUE SOUMISES A UNE DIGESTION ENZYMATIQUE PAR ACTION DE RIBONUCLEASE.

J. GIUNTINI et Y. T. TCHAN, Institut Pasteur, Paris.

On admet aujourd'hui que la structure interne des bactéries comporte un appareil nucléaire, et des études biochimiques le font considérer comme de nature désoxyribonucléique. Malgré les perfectionnements techniques apportés aux colorations et aux digestions, l'étude cytologique de cette question conduit à l'aide du microscope ordinaire reste peu précise, l'appareil nucléaire étant de l'ordre de grandeur du pouvoir séparateur, 0,25 μ, du microscope optique en lumière visible. Nous avons donc pensé pour faire de nouvelles investigations à utiliser le microscope électronique dont le pouvoir de résolution est de quelques mμ.

Plusieurs auteurs, par différentes voies, se sont déjà occupés de mettre en évidence la structure des bactéries au moyen du microscope électronique. Knaysi et Mudd traitent les préparations au bicarbonate de sodium, Knaysi fait germer les spores de *B. mycoides* en milieu sans phosphore ni azote et rend ainsi le cytoplasme plus transparent que le noyau, Robinow et Cosslett montrent que par examen direct l'appareil nucléaire diffracte moins les électrons et apparait plus clair que le cytoplasme. J. Hillier, Mudd et Smith confirment, à l'aide d'un dispositif augmentant le contraste de l'objectif, ce dernier fait. Mais, si l'observation au microscope électronique de l'appareil nucléaire est gênée par les acides nucléiques, il nous a semblé logique d'utiliser des techniques pour enlever l'un des deux acides pour rendre l'autre visible, le noyau étant composé principalement d'acide désoxyribonucléique. La digestion chlorhydrique à 60° présente des difficultés techniques pour les supports d'objets destinés à être examinés au microscope électronique; aussi nous sommes nous adressés à une digestion enzymatique à ribonucléase qui offre des garanties de spécificité et respecte la membrane en collodion.

En pratique, la technique présente quelques difficultés, l'aspect au microscope électronique dépendant avant tout de la diffraction relative des diverses parties de la cellule. Une préparation digérée pouvant être parfaite à l'examen au microscope ordinaire, reste absolument homogène sans aucune structure interne au microscope électronique. Il nous a donc fallu diminuer une quantité importante d'acide ribonucléique pour que les électrons puissent révéler la structure des ap-

pareils nucléaires et réaliser un titrage de digestion pour obtenir des préparations convenables. La technique est la suivante: on met en suspension dans l'eau physiologique une culture en pleine croissance (phase logarithmique), on dépose une goutte sur la membrane du porte-objet, on laisse sécher et on fixe au fixateur Chabaud pendant 1 heure puis on digère à 37° de 5 en 5 minutes en procédant à un véritable titrage de l'enzyme sur les préparations. Une fois la digestion terminée, on lave à l'eau distillée durant une heure. Ces préparations sont alors soit observées directement au microscope électronique, soit après un ombrage à l'or. Nous allons examiner quelques résultats obtenus par cette technique sur des bactéries pathogènes se répartissant en coccus et en bâtonnets Gram positifs et Gram négatifs.

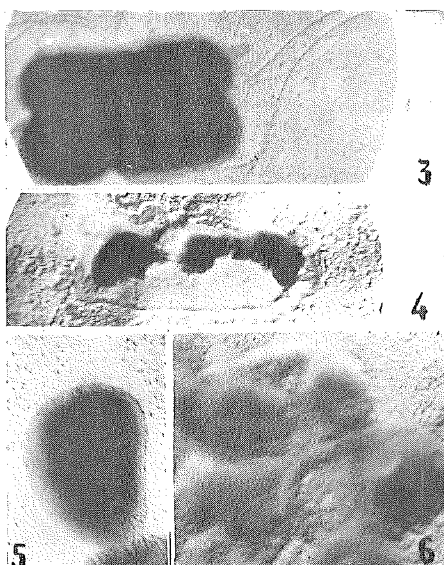

Fig. 2. *Escherichia coli*
3 non digérés
4 digérés.
Vibrio cholerae,
5 non digérés,
6 digérés.

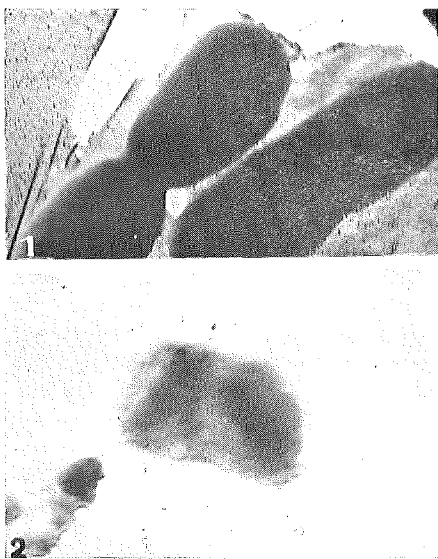

Fig. 1. *Pasteurella pestis*,
1 non digérés,
2 digérés.

1. Pasteurella pestis (Fig. 1, images 1 et 2). Les cellules jeunes de ces germes sont presque complètement opaques aux électrons (accélération 80 kV). Une fois digérées, on voit les deux masses nucléaires soit à chaque extrémité de la cellule, soit vers le centre; la position des masses nucléaires confirme les observations antérieures faites par nous a microscope ordinaire. En plus, on voit nettement que la structure des masses nucléaires est hétérogène, l'interprétation de cette hétérogénéité restant encore très difficile.

2. B. coli (Fig. 2, images 3 et 4). Nous avons pu mettre nettement en évidence les appareils nucléaires dont les images correspondent bien à celles obtenues en lumière visible; leur structure est hétérogène.

3. Vibro cholerae (Fig. 2, images 5 et 6). Le vibrion cholérique jeune est déjà perméable aux électrons pour pouvoir l'examiner en microscopie électronique sans digestion. Cependant les images obtenues après digestion nous permettent de mieux situer l'emplacement des désoxyribonucléoprotéides.

4. Streptococcus mastitidis (Fig. 3, images 7 et 8). On distingue à l'intérieur des coccus, après digestion, deux demi-sphères à bords arrondis, symétriques par rapport au plan de séparation. D'autre part après la fin de la division, les deux cellules filles semblent collées par leur appareil nucléaire d'une façon plus ou moins lâche, ce qui expliquerait l'accolement en chainettes.

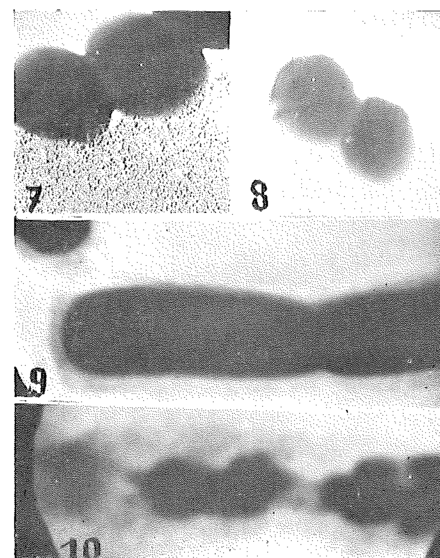

Fig. 3. *Streptococcus mastitidis*.
7 non digérés,
8 digérés.
Bacillus anthracis,
9 non digérés avec capsule,
10 digérés.

5. Bacillus anthracis (Fig. 3, images 9 et 10). L'appareil nucléaire du *B. anthracis* se voit très nettement après digestion. Il est hétérogène et correspond aux observations de Flewetts en lumière visible.

Conclusion. Il nous semble que ce travail apporte une certaine précision sur l'appareil nucléaire des bactéries, et par sa correspondance avec les études cytologiques des bactéries en microscope en lumière visible, confirme la présence de masses nucléaires chez les cellules jeunes en pleine croissance.

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Discussion.

Dr Gregoire: With Dr M. Welsch we performed the same kind of experiment. We found that cells fixed by Chabaud's fluid were very much damaged when examined with the electron microscope. Treatment of fixed bacteria at 37°, 56° or 65° with purified but not crystalline ribonuclease allows good demonstration of the nuclei by Giemsa's method. However, with the electron microscope, clearing of the cytoplasm was observed but no definite correlation could be assumed between light and electron microscope pictures.

Réponse: Nous n'avons pas observé de déformations importantes après la fixation par le fixateur Chabaud.

La digestion par la ribonucléase est faite à 37° dans une chambre humide. Pour obtenir des images nettes des appareils nucléaires des bactéries, il faut effectuer un véritable titrage de l'enzyme comme l'indique notre publication. Il n'y a pas de règle absolue, le temps de digestion et la concentration en enzyme varient suivant les bactéries.

Nous avons observés une certaine correspondance entre les images obtenues par le microscope en lumière visible avec celles obtenues en microscopie électronique.

Mr Hedin: What was the temperature at which te digestion took place? Dr Wyckoff and I have observed that owing to artefacts one must be extremely careful in evaluating results obtained on material which has been heated over say 50° C. I also want to ask

about the specificity of your preparation of ribonuclease: was it for instance entirely free of proteolytic activity?

Réponse: Le digestion est faite à 37° C. L'enzyme utilisé est un ribonucléase pancréatique cristallisé et chauffé au préalable à 100° pour détruire les desoxyribonucléase. Sa spécificité est considérée par nous comme satisfaisante.

Mr van Duijn: Are the structures obtained by staining with the light microscope the same as those obtained by electron microscopy?

Réponse: Il y a une certaine analogie entre les images obtenues par le microscope en lumière visible et le microscope électronique. Cependant on peut espérer apprécier des détails de structure grâce au grand pouvoir de résolution du microscope électronique.

COMPARATIVE PHASE CONTRAST AND ELECTRON MICROSCOPE STUDIES ON ANIMAL CELLS.

W. BERNHARD and H. MANGINI, Institut du Cancer, Villejuif (Paris).

There are different procedures for examining cells in the electron microscope.

1. A cellular suspension can be brought on the membrane of the grid.

2. The *replica technique* recently applied with good results by Claude.

3. By means of *sections,* probably soon a more current method (Bretschneider).

4. The tissue culture. The advantage of this technique: The cells having grown directly on the plastic membrane are well spread out and the border zone of cytoplasm is thin enough to be penetrated by electrons. Porter, Claude and Fullam have developed this most important method, which gives best results and which we used for our own studies.

Little fragments of tissue are put on a cover-glass covered itself with a formvar film and allowed to grow in a clot of fibrin and nutrient solution in the classical way. A lot of new cells remain on the membrane, when the clot is removed. Then the formvar film is carefully drawn off the glass and put on a disc. In this way we examined heart fibroblasts of chicken embryos and Rous sarcoma cells.

To get an idea of possible artefacts, we thought it useful to compare electron microscope patterns with pictures of living cells. Porter, Claude and Fullam, having compared stained fibroblasts in the ordinary microscope with electron micrographs, made the important statement, that cells when brought in the electron microscope conserve their natural form without changing very much. For special investigations, however, it seemed useful to make a photograph of part of a living cell, then, after special treatment, to make an electron micrograph of the same part, in order to compare identical structures.

We used a Phase contrast microscope for the study of the living cells, which were observed either directly through the clot, or after removal of the latter. The remaining cells, rinsed with Tyrode solution and covered with a cover-glass, were now examined and one of them, easily recognizeable by a characteristic shape, was photographed. Next the preparation was treated according to the technique as used by the authors named above: Fixation with vapors of osmium tetroxyde 5′ to 20′, cleaning in distilled water 10′ to 30′. Then the formvar film was half drawn off the glass under water

and a grid was put between this and the interesting part of the membrane. All this has to be controlled with a microscope of low magnification. A higher magnification was now used to get the interesting cell over a central square of the grid. For this, we lifted the glass with the film out of the water and by means of a micropipette we moved the thin layer of water, which always remains between glass and membrane. So, without touching the latter, we carefully floated the cell in the direction we wanted. Without using a micromanipulator the finest movements may be achieved in this way.

The specimen was desiccated in the usual manner with phosphorpentoxyde. Fig. 1 and 2 show a sector of the same Rous sarcoma cell, 1 is situated more central, 2 a bit more in the periphery.

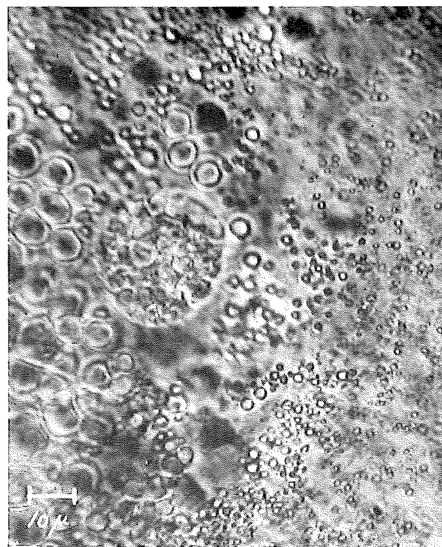

Fig. 1. Phase contrast picture of unstained cell. In the cytoplasm a lot of little rings of different diameters may be seen. These correspond to the fat granules in Fig. 2, which are forming diffraction circles if they are beneath or upon the optical plane of observation. Big vacuoles and a nucleus are still to be seen.

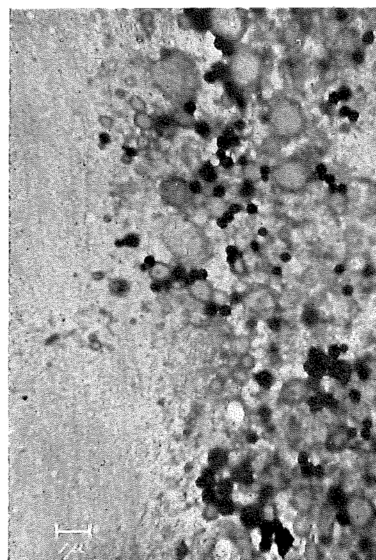

Fig. 2. The electron micrograph shows much more details: Cell border, ectoplasm with chromosomes, endoplasm with the black fat granules and vacuoles of all diameters. Some of them derive probably from mitochondria; no chondriconts are visible on this part. 48 kV. Trüb Täuber E.M.

This example shows the possible comparison of the same element alife and in the electron microscope. To control the formation of artefacts and to observe different functional states of the cell this may be a useful method.

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Discussion.

Dr Cosslett: This comparison is very important, giving a control of the reliability of electron micrographs of dried biological material. We have begun such work with *Mycobacterium phlei* and find a remarkable similarity between electron microscope and phase contrast pictures. This gives the possibility of detecting drying artefacts.

Dr Locquin: 1°) Nous pensons que l'absorption de la plaque de phase de votre microscope était trop faible pour rendre les plus fins détails.

2°) L'examen d'un objet "shadowcast" donne des images très semblables a celles qu'on obtient au microscope électronique.

Réponse: Nous ne pensons pas que notre microscope soit mauvais. Le contraste dans quelques-unes de nos cellules projetées est un peu faible, mais nous avons déjà fait un choix. Les couches minces de protoplasme donnent un contraste inférieur à celui qu'on pourrait obtenir avec des cellules plus épaisses, qui seraient alors trop épaisses pour l'examen au M. E.

Dr Cuckow: Has Dr Bernhard confirmed the earlier observations, that electron micrographs of these Rous sarcoma cells grown in tissue culture show the presence of colonies and of single dense particles agreeing in size with that of the Rous agent as determined by filtration?

Answer: We have some pictures wich are suspect, but they don't allow us to confirm the observation by Claude, Porter, and Pickels. Perhaps our technique is not yet perfect and we shall go on.

Miss Van Iterson: How do you manage to get your selected part exactly on the grid, and do you study the living specimen on the slide first, or do you study it on the grid?

Answer: To be sure that we observed intact cells, we avoided all unnecessary manipulation and studied the cells on the slide first.

A CONTRIBUTION TO THE KNOWLEDGE OF THE SURFACE STRUCTURE OF WOOL.

BO PHILIP and GÖSTA LAGERMALM,
Swedish Institute for Textile Research, Göteborg, Sweden.

In recent years electron microscopy and improved optical microscopy have led to an advanced knowledge of the histology of wool and other animal fibres. The following is, with the exception of some historical notes, a short survey of the work carried out in this field at the Swedish Institute for Textile Research. This work has been done under the guidance of Professor Nils Gralén and among other collaborators we must mention Mr Joel Lindberg, who has taken considerable part in the investigations, and Dr E. H. Mercer, who made many helpful suggestions.

The characteristic scale-structure of wool is easily visible in the optical microscope. Allwörden [1] observed that small blisters were formed on the surface of the fibre when virgin wool was treated with chlorine water. In some places these blisters united and formed bigger ones, frequently covering several scales. The Allwörden-reaction has been used as an indication if the wool has been alkaline damaged or not. Whewell and Woods [2] studying the dyeing properties of wool fibres made the suggestion that a thin membrane might cover the fiber surface. Other research workers as Reumuth [3], Lehmann [4, 5], Elöd and Zahn [6] formed the conclusion that there existed a membrane, lying underneath the scales. In 1948, when the residue from resin-treated wool fibres, which had afterwards been dissolved in Na_2S, was investigated, very thin membranes were observed [7]. These membranes, however, were found even in the residue from untreated fibres and accordingly they had to be interpreted as a constituent of the wool itself. The membranes seemed to be about 100—200 A.U. thick, smooth and nicely folded. They are very resistent to chemical treatments, which makes it very probable that they do not consist of an ordinary protein or keratin. By careful chlorinating followed by washing and shaking with water the same folded membranes could also be obtained. From this it could be understood that the membrane must be located on the surface of the fibre and also that we had to do with the semipermeable membrane responsible for the Allwörden blisters. As this membrane lies on the surface of the cuticle, it is called the epicuticle, in analogy to the customary nomenclature of histological tissues. [8]

Lehmann [5], Elöd and Zahn [6] had treated wool with formaldehyde or phenol, in the latter case followed

by digestion with enzymes. Fig. 1 shows a specimen obtained with phenol treatment followed by trypsin digestion. As earlier mentioned Zahn is of the opinion that the material isolated by the action of trypsin is a membrane lying underneath the scales. There have, however, been no difficulties in isolating the epicuticle from the phenol treated preparations [9] by chlorination of the tubes. The most probable explanation is that right under the epicuticle there is a second layer, which is hardened by the phenol and fastened to the epicuticle. This second layer, the exocuticle, is shown by Mercer and Rees [10] to be digestible by enzymes like trypsin. That the Lehmann tubes (from the formaldehyde treatment) consist of the epicuticle + exocuticle and perhaps even part of the scales, can be shown by dissolving the tubes in Na_2S [9]. Only the epicuticle will be left. When treating the fibres with 1-m NaOH the last part being dissolved, besides the epicuticle, is the exocuticle. The exocuticle may be regarded as a cementing substance which fastens the epicuticle to the underlying main part of the scales, the endocuticle. Schematically the cuticle can be divided into three layers: on the surface there is the epicuticle which has a thickness of the order of magnitude 50—200 A.U., underneath it lies the exocuticle which fastens the epicuticle to the under-lying trypsin resistant endocuticle which possesses an uneven surface which can be observed after etching with trypsin [10].

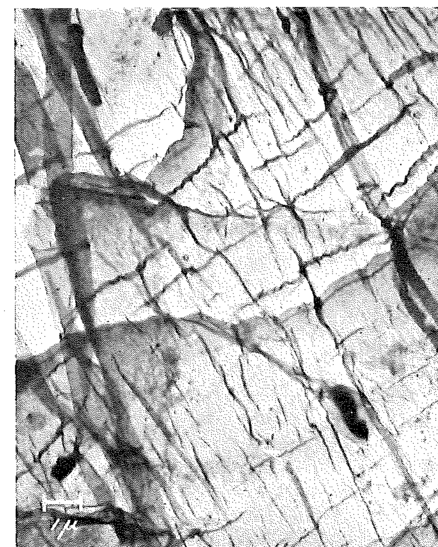

Fig. 1. Specimen obtained with phenol treatment followed by digestion with trypsin. Transmission.

The thinnest and most homogeneous preparations have hitherto been obtained by the method mentioned where the wool is dissolved

Fig. 2. Epicuticle obtained by treating the wool firstly with alcoholic KOH and afterwards dissolving it in Na_2S. Goldshadowed.

in Na_2S and where the epicuticle is left as the most resistent part of the material. This reaction was originally carried out at room temperature. Then about two months were necessary to produce the clean epicuticle. By increasing the temperature to about 50° C it has been possible to reduce this time to about one week. Figures 2 and 3 are Au shadowed pictures prepared by treating the wool firstly with alcoholic KOH and afterwards dissolving it according to the method mentioned above. The treatment with alcoholic KOH is, however, irrelevant in this connection as the same results have later been obtained merely by dissolving the wool in Na_2S. In Fig. 2 the epicuticle exhibits a rougher surface than has previously been noticed. The thickness varies from point to point and often exceeds the values to be expected for exocuticlefree epicuticle. This may indicate a loosening of the structure thus making the epicuticle look thicker. Fig. 3 shows a still more affected part of the same specimen. Here it can be observed that the epicuticle has been perforated and is breaking up into smaller particles or rods, but still the continuous membrane-like structure is quite noticeable. Actually this alkaline damage has already been indicated in the first published pictures of the epicuticle [7] although the evidence is not so striking in this first result. The action of alcoholic KOH, which considerably increases the dyeing velocity of wool, may be explained in a similar way. The reagent probably perforates the epicuticle thus giving the dye molecules free admittance.

The alkaline damage indicates that the epicuticle may consist of two components, the one sensitive to alkaline treatment, the other not so sensitive, forming some kind of skeleton structure that finally is split up into isolated particles. If we have to do with two components, the one dissolved cannot possibly be the same substance cementing the epicuticle to the rest of the fibre. The latter is dissolved by the chlorine, while, at the same time, the epicuticle is not perforated but instead blistered by the Allwörden reaction.

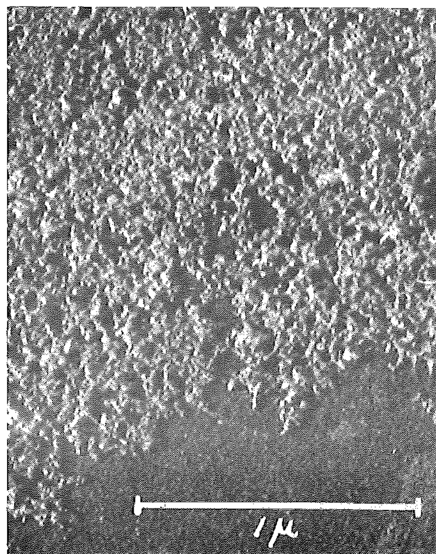

Fig. 3. Epicuticle, from the same preparation as the one shown in Fig. 2.

At the present stage too wide conclusions should not be drawn regarding the constitution of the epicuticle. Its constitution and chemical behaviour will be further investigated at the Swedish Institute for Textile Research.

Acknowledgement. One of us (Lagermalm) would like to express his thanks to the International Wool Secretariat for granting him a studentship for further investigation of the epicuticle.

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Discussion.

Ir Nieuwenhuijs: How did you come to the idea that there might be a thin epicuticle?

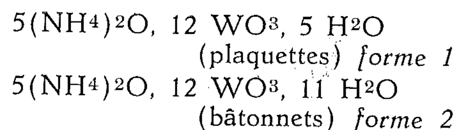
Answer: The epicuticle was occasionally first observed in trypsin-digested wool fibres. No notice however was paid to this until the same phenomenon was observed in the residue from melamine-resintreated wool dissolved in Na_2S. This made us suspect that they were a constituent of the wool itself and initiated the later investigations.

PRECIPITATION DU PARATUNGSTATE D'AMMONIUM EN MILIEU EAU-ALCOOL.

Mlle A. E. MATHIEU SICAUD,
Laboratoire Central des Services chimiques de l'Etat, Paris.

En vue d'obtenir des suspensions microcristallines dispersées, nous avons étudié au microscope électronique la précipitation du paratungstate d'ammonium.

Selon Smith et Exner le paratungstate se présente sous deux formes:



$5(NH_4)_2O$, $12\ WO_3$, $5\ H_2O$ (plaquettes) *forme 1*

$5(NH_4)_2O$, $12\ WO_3$, $11\ H_2O$ (bâtonnets) *forme 2*

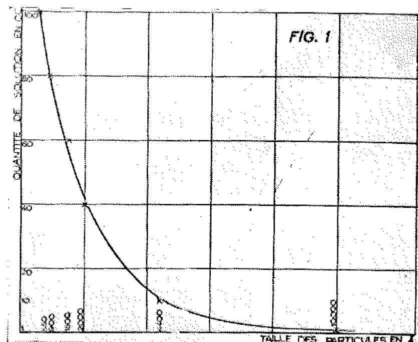
Ce corps a été choisi par suite de sa faible solubilité dans l'eau. La précipitation par l'alcool donne alors des particules très petites.

La solution saturée de paratungstate dans l'eau est versée aussi vite que possible dans 200 cm³ d'alcool éthylique à 0° en agitant fortement le mélange.

I. Influence de la variation de la quantité de solution. Ceci revient à faire varier la quantité de germes cristallins introduits dans l'alcool (précipitant). Les particules obtenues sont des plaquettes quadratiques minces (forme 1).

La courbe 1 (Fig. 1) correspondant à un alcool à 96° montre le changement de la taille moyenne des particules en fonction de la quantité de solution introduite.

La taille moyenne des particules pour toutes les expériences a été établie statistiquement par les méthodes classiques sur environ 300 particules.

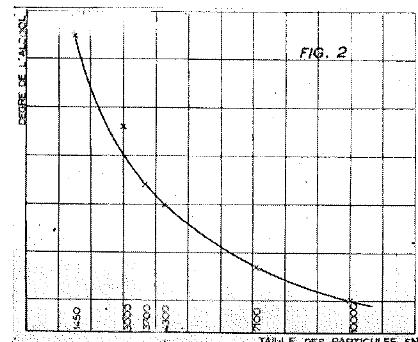


La diminution de taille des particules s'accompagnant d'une déformation importante du microcristal nous avons fixé la quantité de solution à 10 cm³ pour 200 cm³ d'alcool. Ceci donne alors des cristaux de taille minima encore bien formés.

2. Variation du degré de l'alcool. Ceci équivaut à modifier l'efficacité du précipitant.

La courbe 2 (Fig. 2) représente la variation de la taille moyenne des particules en fonction du degré de l'alcool. Pour un alcool assez riche en eau le précipité est constitué d'agrégats en forme de croix d'environ 1 μ (superposition de plaquettes), lorsque la teneur en eau diminue les agrégats disparaissent et pour un alcool presque absolu on n'obtient plus que des plaquettes très fines.

Après centrifugation, la suspension contient seulement des particules de 1000 U.A. à quelques % près (Fig. 3). L'épaisseur mesurée par shadow casting est de 300 U.A.



3. Vieillissement des suspensions. Cette étude a été faite pour un alcool à 96°4.

Lorsque la suspension est fraîche, elle est constituée exclusivement de plaquettes.

Au contraire pour une suspension âgée d'environ six jours on n'observe plus que des bâtonnets (Fig. 4).

Nous avons alors effectué des prélèvements dans la suspension en fonction du temps et l'observation au microscope électronique nous a permis de suivre la transformation.

Au bout de 6 heures les plaquettes ont formé des agrégats en croix très analogues à ceux observés pour un alcool très humide.

Au bout de 23 heures les agrégats sont devenus compacts et on observe la croissance de bâtonnets à partir de leurs bords externes.

Les bâtonnets croissent avec le temps et deviennent de plus en plus nombreux. Le Fig. 5 correspond à 26 heures. Tous les intermédiaires ont été ainsi observés entre plaquettes et bâtonnets. Ces derniers digèrent les particules par un phénomène assez voisin de celui observé lors de l'hydratation du ciment.

Le microscope électronique a permis de suivre facilement une transformation très difficile à mettre en évidence par un autre moyen. Il a été possible d'observer le passage au cours du temps du paratungstate d'ammonium à 5 molécules d'eau, au paratungstate à 11 molécules d'eau.

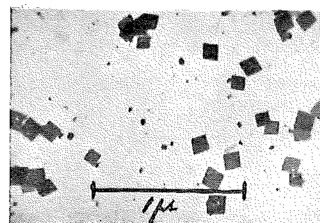


Fig. 3.

Fig. 4.

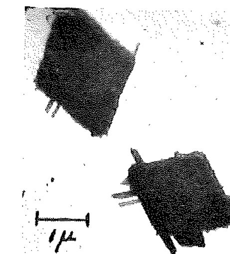


Fig. 5.

CREEP OF STEEL AND ELECTRON MICROSCOPE *)

P. COHEUR and L. HABRAKEN, Centre National de Recherches Metallurgiques, Université de Liège.

The purpose of this paper is to give the first results obtained by our laboratories in our researches with the Electron Microscope on Cr-Mo steels during creep tests.

The steels used in the investigations have the following approximate composition:

— Cr 2 per cent;
— Mo 1 per cent;
— C 0,1 per cent.

The creep has been effected at 550° and under a stress 16 to 19 kgs/mm². Prior to creep, the steel has been normalized at 925° and stabilizing annealed at 775° for 6 hours.

For the electron microscopic examination we have used the classical process of formvar replica shadowed with Cr. The electronic magnification is 4000 x and the photographic magnification 2 to 2.5 x.

a. The original states of our steels after the heat-treatment we have just described, are fairly similar.

We observe (Fig. 1) precipitates more or less spheroïdal, scattered in the grain boundaries, and inside the grains.

The inclusions inside look smaller than those at the boundaries.

b. After annealing for the same time and at the same temperature as the ones corresponding to the creep test, that is to say at 225° C below (so to speak) the stabilizing temperature we observe that the inclusions of some of our steels are growing and aggregate at the boundaries.

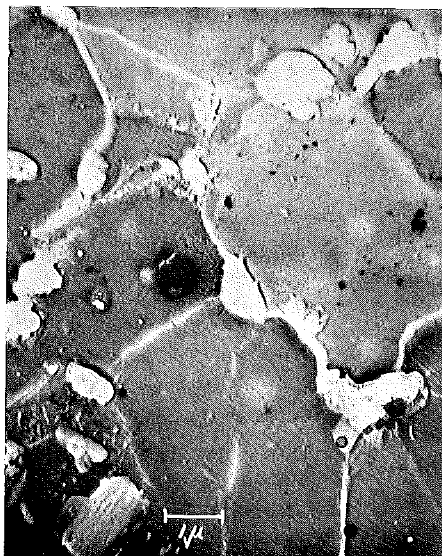


Fig. 1.

*) Published under the auspices of the „I.R.S.I.A." (Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture).

For other steels, on the contrary, we observe that the size of the precipitates has decreased from the original state, but their density has increased very definitely, both at the boundaries and inside the grains (Fig. 2).

ture is 225° lower than the temperature of the initial tempering.

c. Now, if we examine the steel after the creep test we observe, at first, that the grains have undergone a more or less important plastic deformation. Sometimes, we observe new precipitates which are different from the previous ones by their elongated shape and their aligned formation. The alignment of these precipitates seems to indicate that they are situated in slip planes and are caused by creep-strains.

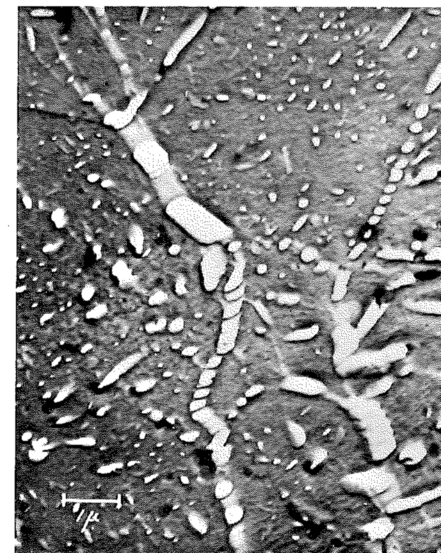


Fig. 2.

Incidentally, the creep properties of these steels are different.

In order to confirm our observations, we have isolated the carbides of one of the last steels referred to by the iodine method and analysed them by X-Ray diffraction.

We found that the steel, after annealing at a rather low temperature, contains, not only the carbides of the steel in the original state, but also at least one kind of a new carbide which was not present in the original structure.

It follows that a new carbide, or a new phase appears in the annealing at 550° although this tempera-

Examined, as before, by X-ray diffraction, the carbides of this steel do not elicit any different phase from that which we had found after annealing at 550° C. Therefore, either the new elongated precipitates have the same structure as their neighbours or they are too scarce to be detected by X-ray diffraction.

Sometimes we also observe inside the slip-lines (Fig. 3) and in the twin planes, very fine precipitates probably originated by the severe strains which are localized in them. Moreover we observe that the slip lines cross strips of a twisted shape,

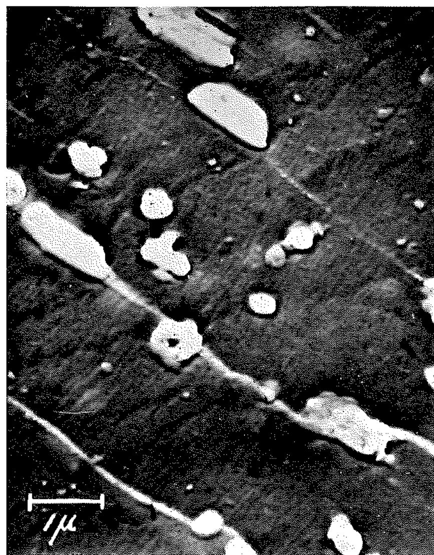


Fig. 3.

indicating the importance of the disturbances undergone in the lattice. These disturbances could be the cause of the breaking of the grains.

Finally, some grains in certain steels show a very definite broken state which might be related to the "veining".

The study is still proceeding but our present results may be summarized as follows; our observations show, that the carbides originated in the heat-treatment are not necessarily stable at the creep-test temperature, although this temperature is 225° C lower than the temperature of the initial tempering.

Our observations show also that the tensile strengths play a certain part in the evolution of carbides.

Particularly, we notice in the parts of the metals, submitted to strains and in fact for definite rates of stresses, new constituents characterized by their elongated shape and their orientation.

Finally during the creep the grains may be broken in many fragments.

Discussion.

Dr Rathenau: Do you think that in your experiments the different stages of creep such as defined by Andrade, namely transient creep, and viscous creep can be separated? The flow lines, along which carbide precipitations are to be seen, probably correspond to transient creep.

Answer: We cannot tell much because the samples which have been examined and had undergone some creep were in the viscous state. Actually we are studying samples in their various states of sollicitation and we hope to be able to give you pretty soon some results.

RECHERCHES AU MICROSCOPE ELECTRONIQUE SUR LES PREMIERS STADES DU DURCISSEMENT D'UN ALLIAGE ALUMINIUM-CUIVRE.

R. CASTAING, Laboratoire de l'O.N.E.R.A., Paris.

Les modifications de structure d'un alliage aluminium-cuivre au cours du revenu à diverses températures ont fait l'objet d'études détaillées utilisant les rayons X et le microscope métallographique ordinaire. Elles présentent en effet une grande importance en métallurgie puisqu'elles sont responsables du phénomène de durcissement structural de ces alliages.

Le microscope ordinaire permet de déceler dans des échantillons soumis à un revenu à plus de 150° la présence d'un précipité formé de plaquettes orientées parallèlement aux plans (100) de la matrice; c'est-à-dire parallèlement aux faces du cube du réseau de l'aluminium. La forme et l'orientation des précipités ne sont cependant discernables au microscope ordinaire que pour des dimensions de l'ordre du micron. Ce précipité constitue ce qu'on appelle la phase Al_2Cu Θ' de Wassermann et Weerts. Les rayons X permettent d'autre part de constater, avant l'apparition de la phase Θ' la présence de zones de rassemblement d'atomes de cuivre (zones de Guinier-Preston) qui se transforment au cours du revenu en zones à surstructure.

Le microscope électronique était tout indiqué pour établir la jonction entre les deux techniques et nous avons fait une étude comparative, aux rayons X et au microscope électronique, sur des échantillons d'alliage aluminium-cuivre à 4 % aux différents stades du revenu.

Nous avons choisi la méthode de l'empreinte d'oxyde: les échantillons étaient polis électrolytiquement au bain perchlorique-acétique, puis soumis à l'oxydation anodique sous une tension de 24 V dans une solution saturée de phosphate d'ammonium.

Nous allons rapidement passer en revue les aspects micrographiques correspondant aux diverses structures de l'alliage, telles qu'elles sont déterminées par les rayons X.

Les revenus à température comprise entre 150 et 200° font apparaître sur le diagramme de rayons X la phase Al_2Cu Θ'. Les micrographies électroniques montrent, dans ce cas, une répartition dense et uniforme de précipités en forme de plaquettes en épitaxie sur le réseau de la matrice. Leur épaisseur moyenne est de 100 U.A. et reste sensiblement constante pour des revenus relativement longs à température inférieure à 250°, tandis que s'accroissent les dimensions latérales des précipités,

beaucoup plus rapides que les trempes douces comme la trempe à l'huile ou les trempes étagées. Ce phénomène est d'ailleurs complètement vérifié par l'expérience.

Discussion.

Dr Brown: Has the appearance of the platelets been correlated with increasing hardness for one usually finds that hardening is complete and even over-complete before visible platelets appear; the hardening being due to zone formation, increase in size of platelets should reduce the amount of copper available for zones and thus perhaps lead to a slight softening.

You are certainly to be congratulated on obtaining precipitation on boundaries of polygonisation.

Réponse: La phase Θ' n'est pas responsable du durcissement. L'irrégularité de la précipitation est cependant telle que certaines plaquettes sont déjà formées avant que la dureté maxima soit atteinte.

Mr Trotter: Do I understand that the last four or five slides were structures as formed at room temperature?

Réponse: Les derniers clichés correspondent à un vieillissement à 150°. Nous n'avons rien observé sur les échantillons vieillis à la température ordinaire.

E. M. RESEARCH IN THE OIL INDUSTRY.

T. REIS, French Petroleum Institute, Paris.

The aim of this paper is to present some results, concerning the oil industry, that we obtained at the French Petroleum Institute with an electrostatic microscope. I am aware of the fact that these results are relatively poor and I apologize for presenting them. But in spite of the fact that important work in this field has been achieved in other countries relatively few papers have been published. We thought that presenting a paper on our work might stimulate discussions and further publications.

We distinguish between two types of observation:

A. on Shape and Size by direct transmission, and

B. on Surface Structure by replica techniques.

A. Shape and Size.

1. Surface calculations.

Various types of powders can be examined. It is obvious that good dispersion is needed in order to count the particles and to measure their size. The data, derived from the micrographs may be used for size distribution curves. Subsequently numerical tables may be dressed like table 1, which concerns a carbon black powder. The number of counted particles and their mean diameter are given. For a given shape of the particles it is possible to calculate the surface of a powdered material. The results obtained in this field are only in qualitative agreement with those obtained by low temperature adsorption and X-ray diffraction. The agreement depends on the porosity of the studied material. For carbon blacks obtained by hydrocarbon combustion and studied in our laboratory the electron microscope gave always the lowest values. The adsorption method gave an intermediate value between the data obtained by X-ray diffraction and those obtained by electron microscopy. The electron microscopic method for surface measurements has the advantage of taking little time.

2. Clays.

The part clays play in oil industry goes from drilling mud to catalyst supports. The available clays are evidently not all fit for the different uses. For instance we examined a clay of the kaolinite type forming thin sheets of 5 mμ minimum thickness and containing lumps of iron oxide-like material which are clearly visible on the electron micrographs. This clay is not suitable for any use owing to its low adsorption and base exchange capacity. Other studies

made on bentonites and montmorillonites gave good agreement with results obtained from X-ray measurements [1].

The beautiful pictures of diatoms are well known. In our laboratory some diatomeous earth which is found in a shale oil deposit was examined. The organic material was clearly visible in the electron microscope.

Some samples of American drilling mud of unknown composition showed very typical attapulgite rod like character.

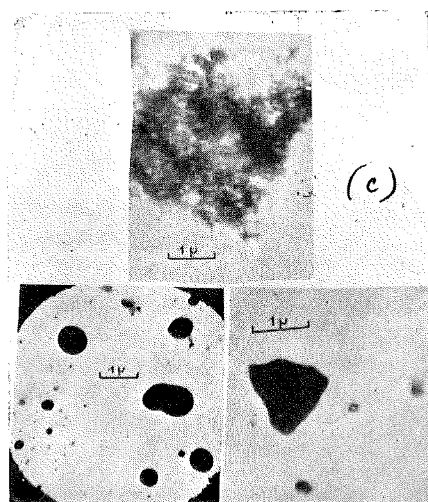


Fig. 1, *a*, *b* and *c*.

3. *Catalysts* [2].

The importance of catalysts need not be outlined here. A fresh catalyst on a clay support shows to have a much bigger area, due to the presence of small particles, than a used catalyst, where the small particles have disappeared [1].

The porous structure of an iron oxide base catalyst is beautifully visible. Very good dispersion was obtained by using ultrasonics [3].

4. *Greases.*

Several papers were published lately on this subject in Holland and in other countries (*a.o.* Ferrington-Birdsall [4] and Hattiangdi-Swerdlow [5]). The results obtained by these authors concern mainly pure products. We studied some commercially available greases: short fiber calcium base grease; the fine fiber structure of lithium base grease on which we observed the presence of some charge material; the twisted fibril structure of long fiber calcium grease, *etc. etc.*

5. *Lubricating oils* [6].

When studying some used lubricating oils we succeeded in separating the metallic particles (Fig. 1*b*) and the carbon black like organic material (Fig. 1*c*) from the initially emulsified phase as observed in Fig. 1*a*.

6. *Carbon black.*

Very characteristic pictures of carbon black, prepared by hydrocarbon combustion, have been obtained. The particle size and aggregation is characteristic for each manufacturing process. Even small changes in this process can be detected.

Shale oil and particularly pure hydrocarbons can be studied too. In another communication some interesting pictures of a hydrocarbon containing 24 C atoms will be shown.

B. Surface structures.

1. *Corrosion.*

Corrosion is an important subject in the petroleum industry (pipe line, sea line, tubular furnace, condensers, *etc.*).

A classical example is the modi-

fication of an aluminium surface by the attack of an acid. Steel corrosion in sea water was shown to take place at the crystal boundaries [7].

2. *Lubrication.*

The surface damage caused by improper lubrication was studied by electron microscopy [8]. In a study concerning the wear on a steel gear surface it was clearly shown that the wear has a preferential direction. One may even recognize very small pits in the surface.

Specialists in lubrication in our laboratories suppose that these small pits, which have a regular diameter, may be due to very small electric discharges between two steel surfaces when the oil film is too thin.

3. *Research on surface protecting agents.*

Some investigations are made in our laboratories (Prof. Hugel) on the use of hydrocarbon derivatives to form a surface film protecting the metal against corrosion. Fig. 2 and 3 show that the surface films formed depend on the molecular structure of the attacking agent. Fig. 2 shows the clean steel surface before the attack and Fig. 3 shows the characteristic modification of the surface as a result of the attack by a naphthyl mercaptan type hydrocarbon.

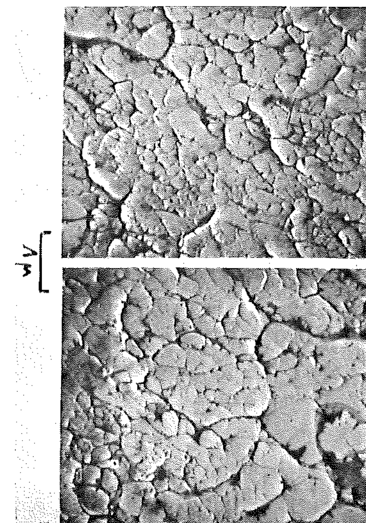


Fig. 3.

The same surface after attack by a different mercaptan shows a very different pattern (Fig. 4).

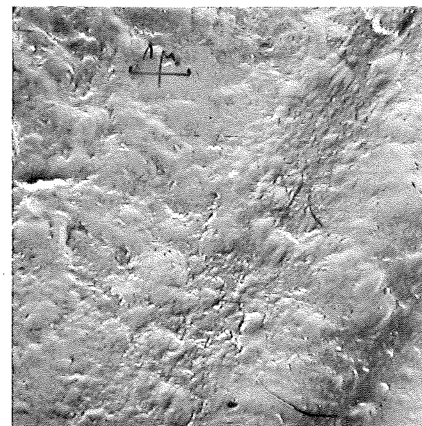


Fig. 2

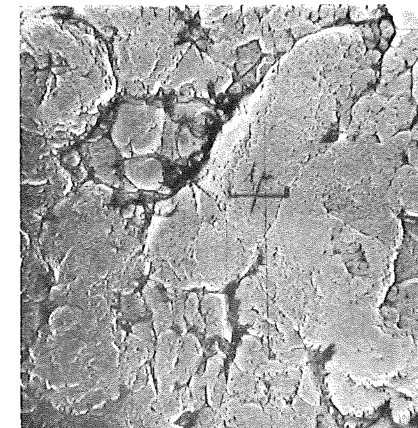


Fig. 4.

A preliminary mass spectrometric study of the desorbed gases from these surfaces seems to indicate the differences in composition between these surface layers.

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THE STRUCTURE OF CORONENE AND ITS MODIFICATION DURING THE OBSERVATION IN THE ELECTRON MICROSCOPE.

T. REIS, French Petroleum Institute, Paris.

1. The structure of coronene $C_{24}H_{12}$.

The structure of the coronene molecule $C_{24}H_{12}$ is of particular interest in view of its high symmetry. The molecule (Fig. 1) consists of 7 benzene cycles and with regard to the electron distribution and bond lengths the structure should be intermediate between benzene and graphite [1, 2]. The crystal structure was studied by Robinson and White [3] by X-ray diffraction. They obtained needle shaped monoclinic single crystals greatly elongated along the *b* axis. The unit cell dimensions were a = 16.1 A.U.; b = 4.695 A.U. and c = 10.15 A.U.; $\beta = 110.8°$. There are two centro-symmetrical molecules in the unit cell. The plane of the molecule is inclined approximately 45° to the *b* axis, as shown in Fig. 2. The perpendicular distance between the molecular planes is approximately 3.3. A.U. (3.41 A.U. in graphite). Robinson and White [3] gave the electron density distribution as projected along the *b* axis on the 010 plane as shown in Fig. 3.

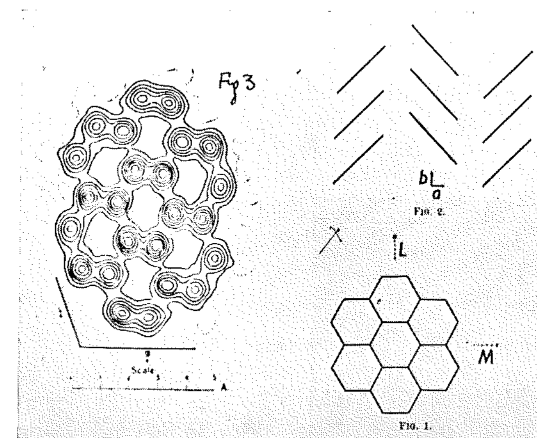


Fig. 2.
Fig. 1.

The crystalline form and especial-

ly the very short *b* axis are similar to those of the phthalocyanines and it was suggested that these large flat molecules might be similarly arranged in the crystals.

The yellow coronene crystals we used came from the I. G. Farben Industrie (Ludwigshafen) and had the following physical properties: Boiling point (at 760 mm. Hg) = 525° C; melting point = 432° C.

2. *Electron microscope observation of coronene.*

The coronene was dispersed in a benzene solution with the aid of ultrasonics. The supporting film was an ordinary collodion film about 150 A.U. thick. During the observation the accelerating voltage and the central electrodes of the two electrostatic lenses were kept at 45 kV and the total emission current was 18 μA for a filament heating current of 1.5 A, and a given geometrical arrangement of the electron gun.

Very remarkable pictures were obtained under these conditions (Fig. 4 and 5). We see that the 4 A.U. thick unit cells form very thin crystal plates of only a few molecular layers which are transparent to the electron beam. The crystalline plates are sometimes normal to the plane of the supporting film and the thicknesses of about 100 A.U. can be directly measured. The thickness of the coronene plates lying on the supporting film, however, is certainly even less.

Fig. 4.

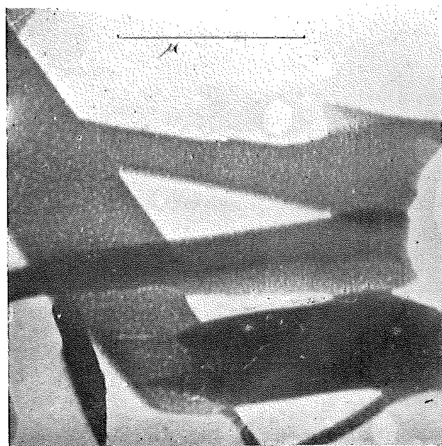


Fig. 5.

It is very interesting to compare our pictures with those obtained by F. A. Hamm and Earl van Norman [4] concerning organic pigments especially the stable form of indanthrene blue R.S. (red shade) after its transformation in the electron microscope.

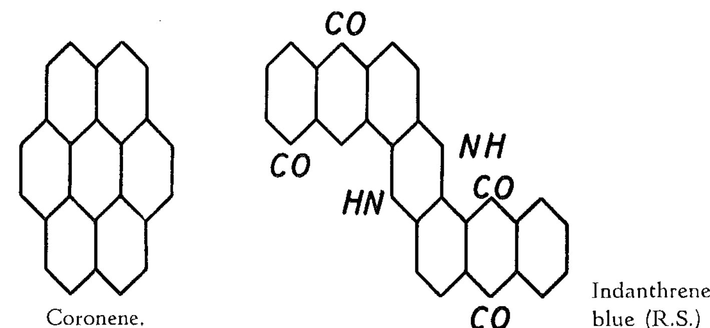
The similarity of the two observed pictures might be due to the structural similarity of these compounds as indicated by their formulae:

The examined copper phthalocyanine gives similar pictures too and the study of other phthalocyanines might be very interesting.

3. *Modification of the coronene in the electron microscope.*

Hamm and Van Norman observed a transformation of the indanthrene blue in the electron microscope and described this as a thermal (polymorphic) or radiation chemical transformation. We tried to do the same with coronene. We know that the electron beam passing through a sample loses part of its energy causing chiefly a heating and an eventual ionisation of the sample. It was calculated that a 10^{-5} mm. thick collodion film irradiated by 55 kV electrons may stand 10^{-3} A/mm^2 owing to its large surface. A film carrying a sample on ⅔ of its surface, however, stands only 10^{-4} A/mm^2. On the other hand ionisation effects have been observed by different authors. Specially beautiful examples are given concerning ionic crystals (Burton, Sennett and Ellis [5]). Using current densities of 1 A/cm^2 in the specimen plane and 40—50 kV, with the specimen mounted on a 100 A.U. formvar film, originally opaque crystals of NaCl, $NaClO_3$ KI *etc.* became transparent under electron bombardement.

In our case when we raised the total emission current of the beam from 18 μA to 22 μA we observed very clearly the destruction of the crystalline structure. In Fig. 5 one sees clearly that the modification of the crystal lattice takes place in a quite regular manner, the resulting "holes" having a quite uniform diameter of about 75 A.U. It seems difficult to explain these changes with the hypothesis of evaporation alone, as no sublimation is observed. Maybe some ionic migration has to be considered or better some preferential *cracking* of the examined organic molecule.



Coronene. Indanthrene blue (R.S.)

One may conclude that the study of the morphology and stability of heavy hydrocarbon molecules by electron microscopy seems to be very promising.

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Discussion.

Prof. Gabor: Do you think what remains is a pure carbon skeleton?

Answer: The diameters of the observed holes are such that they indicate the departure of several molecules. The general crystalline structure is not destroyed. I think that the remaining product is still coronene, but that specific parts of the crystal suffered a preferential evaporation with a possible cracking of the evaporated coronene molecules.

GENERAL TECHNIQUES FOR THE EXAMINATION OF AIRBORNE DUSTS WITH THE ELECTRON MICROSCOPE WITH SPECIAL REFERENCE TO COAL DUSTS.

J. W. SHARPE, Imperial Chemical Industries Research Dept., Widnes, and
R. F. HOUNAM, Medical Research Council, Pneumokoniosis Research Unit, Cardiff.

Introduction.

It is generally agreed that particle size may be an important factor when considering the toxicity of an airborne dust which can cause injury to the lungs. Since a high proportion of the particles present in these dusts may be beyond the limit of resolution of the light microscope, it is necessary to develop techniques which will enable a determination of both the number of particles per unit volume and the size distribution of the particles to be carried out in the electron microscope. The methods described in this paper have been applied successfully to the examination of airborne coal dusts.

Preparation of samples.

The most suitable method for obtaining the necessary samples is by thermal precipitation [1]. In this method the aerosol to be examined is aspirated down a narrow channel, and precipitation of the particles is effected on to suitably disposed surfaces by an electrically heated wire placed centrally across this channel. Only slight modifications to the normal thermal precipitator, as used to obtain samples for examination by ordinary light microscopy, were necessary to enable it to be used for the preparation of electron microscope samples. These involved making two new plugs for the instrument, each having a recess into which a specimen mounting grid could be dropped, and an arrangement for holding the grids flush with the surface of the plugs. By aligning the plugs so that the strip of dust deposited lay parallel to the mesh of the grid, the counting of the particles along a line perpendicular to the strip was greatly facilitated.

The instrument takes samples that can be examined in the electron microscope without further manipulation. The airborne coal dust samples were obtained by setting up the thermal precipitator some 25 yards from the coal face and aspirating a known volume of the air through the instrument. Fig. 1 shows a typical micrograph of a sample of airborne dust from a coal mine, obtained in this way.

Examination of samples.

There are two possible methods of obtaining a size analysis. In the first, the particles are counted and their sizes measured from a number of photomicrographs. This method was found to be tedious, owing to the large number of micrographs required. In the second, a graticule consisting of circles of various known diameters, was marked on a fluorescent screen using an engraving machine fitted with a fine steel cutter. This screen was inserted in the camera cassette of the electron microscope, in the position normally occupied by a photographic plate. When the camera shutter was raised the graticule plate could be used as the final image screen, and particle sizes could be read off directly by matching the particles against the appropriate circles. A note on the construction of the graticule is being published elsewhere. It has been used successfully in size analysis work with a considerable saving in time and labour.

Fig. 1. Typical sample of airborne coal dust.

Normal microscopic methods of particle size determination yield a value of particle diameter based on an estimate of the area of the particle silhouette and this method was adopted in the case of the above size analyses. It is usually assumed that the particles are resting in their most stable position on the glass cover slip, or, in the case of the electron microscope, on the collodion film, and it is assumed that the size of any particle cannot be greater than that estimated.

In order to examine whether this assumption is valid for thermally precipitated samples, several specimens of airborne mine dust collected by the thermal precipitator were shadow-cast with gold/palladium. Fig. 2 shows a sample shadow-cast in this way. The micrographs were subsequently measured and the height of the particles calculated. Results showed that although there may be a slight tendency for particles to be deposited in a position of maximum stability, that is,

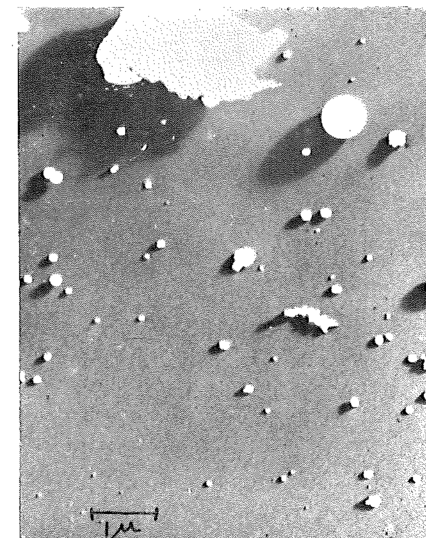

Fig. 2. Shadow-cast specimen of airborne coal dust.

with their least dimension perpendicular to the supporting film, particles can be deposited in most unstable positions (Fig. 3). Hence a close approximation to the true particle size is obtained by the normal methods of counting thermal precipitator records.

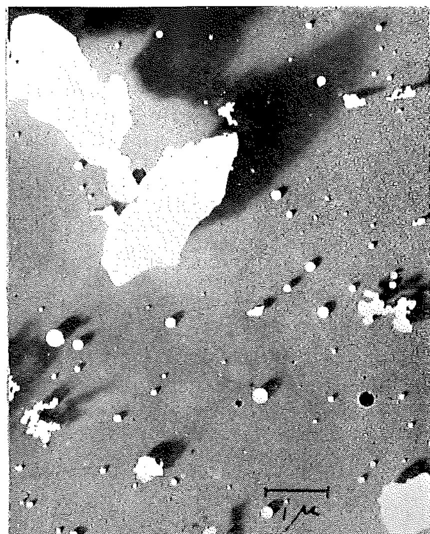

Fig. 3. Shadow-cast specimen of airborne coal dust.

Discussion of results on coaldusts.

Samples of airborne coal dusts were obtained from six collieries, four of which had a low and two a high incidence of pneumokoniosis. The experiments carried out to date have, however, been of an exploratory nature, to determine the best method for routine examination by the electron microscope. It is not yet possible, therefore, to say that there is any difference in either the number concentration or size distribution of the airborne dust particles from mines with a high or low incidence of pneumokoniosis.

At least 75 % by number of the particles visible in the electron microscope were less than 0.2 μ in diameter and, therefore, invisible by light microscope methods. Examination of samples at high magnification has revealed no particles less than 200 A.U., and it seems possible that some of the smallest particles present in the micrographs are ultimate coal micellae.

Reference:

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Discussion.

Dr Brown: It is interesting that no significant difference has been found in distribution of particles between high disease and low disease mines. The suggestion is that the difference may be in the relative number of particles within some small range of sizes (or maybe below a certain limit). This may provide a hard problem for electron microscopy.

Answer by Mr Hounam: It would be unwise on the basis of the few samples yet examined in the electron microscope to state that there is no significant difference in the size distribution of particles from coal mines with a high or low incidence of pneumokoniosis, particularly as a considerable amount of the work to date has been exploratory to determine methods suitable for routine use. The points raised are certainly those to which most attention would be given when an adequate number of samples have been taken and counted by standardised methods.

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