PREFERENTIAL STAINING OF NUCLEIC ACID–CONTAINING STRUCTURES FOR ELECTRON MICROSCOPY

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ABSTRACT

Oriented fibres of extracted nucleohistone were employed as test material in a study of satisfactory fixation, embedding, and staining methods for structures containing a high proportion of nucleic acid. Fixation in buffered osmium tetroxide solution at pH 6, containing 10^{-2} M Ca^{++}, and embedding in Araldite enabled sections of the fibres to be cut in which the orientation was well preserved. These could be strongly stained in 2 per cent aqueous uranyl acetate, and showed considerable fine structure. Certain regions in the nuclei of whole thymus tissue could also be strongly stained by the same procedure, and were identical with the regions stained by the Feulgen procedure in adjacent sections. Moreover, purified DNA was found to take up almost its own dry weight of uranyl acetate from 2 per cent aqueous solution. Strongest staining of whole tissue was obtained with very short fixation times—5 minutes or so at 0°C. Particularly intense staining was obtained when such tissue stained in uranyl acetate was further stained with lead hydroxide. Although the patterns of staining by lead hydroxide alone and by uranyl acetate were similar in tissues fixed for longer times (½ hour to 2 hours, at 0°C or 20°C), in briefly fixed material the DNA-containing regions appeared relatively unstained by lead hydroxide alone, whilst often there was appreciable staining of RNA-containing structures. Observations on the staining of some viruses by similar techniques are also described.

INTRODUCTION

During the past ten years or so, the application of electron microscopy to the study of the cytoplasm of cells, and to the examination of the interrelation between cells, has met with very great success. The widespread use of buffered osmium tetroxide solution as a fixative (Palade, 1952), sometimes followed by additional staining at a later stage according to more personal preferences, has revealed characteristic structures in a wide variety of tissues, whose appearance was consistent with structural information derived from other sources and whose architecture could often plausibly be related to function. Nerve, muscle, motor end-plates, secretory cells, cell membranes, and mitochondria provide well known examples of such correlations. It would be fair to say that most electron microscopists have had reasonable confidence that the available techniques have been giving them a picture of the organization and contents of a considerable part of a cell on which they could place a moderate degree of provisional reliance.

No such confidence has existed concerning the cell nucleus. Its contents appear characteristically granular in tissue sections in most cases, and provide very little in the way of a structural picture of the extremely interesting and important functions believed to be taking place there. It has therefore been rather natural to wonder whether fixation techniques which work satisfactorily on structures containing a high proportion of protein or lipoprotein might be inadequate for structures in which nucleic acids played an important role. Furthermore, it has also seemed possible that even if these nucleic acid–containing structures *were* fixed properly by conventional fixatives, they might still not show up satisfactorily in the electron microscope because of inadequate staining, *i.e.*, because of insufficient uptake of heavy metal by the nucleic acid.

One way of investigating this sort of problem is to use some test substance which contains a high proportion of nucleic acid and is similar in composition to the nuclear contents, and whose fine structure is already known, and to attempt to fix and stain this test substance in such a way that the known structure can be seen in the electron microscope. Unfortunately, there is at present no nucleoprotein whose detailed structure is known, and we have therefore adopted a compromise approach to the problem. A well characterized complex of protein and deoxyribonucleic acid (Zubay and Doty, 1959) known as nucleohistone can be extracted from, for instance, calf thymus tissue, and oriented fibres of this may be prepared by suitable techniques. These fibres give characteristic x-ray diffraction diagrams (Wilkins *et al.*, 1959). The detailed interpretation of these patterns is as yet unknown, but they do show that a characteristic and well oriented structure is present. We therefore treated such nucleohistone fibres as pieces of tissue and endeavoured to fix, embed, section, and stain them in such a way that orientation was still preserved, that the tissue showed up with good contrast in the electron microscope, and that characteristic fine structure, not inconsistent with the x-ray data, became visible.

This approach met with a moderate degree of success and encouraged us to examine a number of aspects of the staining of intact tissues and of various isolated nucleoprotein structures with uranyl acetate and with lead hydroxide. Our observations have been encouraging in so far as they indicate that nucleic acids can be preserved within specimens destined for the electron microscope, that often the nucleic acid–containing regions can be stained in a highly preferential manner, and that under some conditions a differentiation between DNA and RNA may be possible. Some of our results have already been reported briefly (Huxley and Zubay, 1960 *b*, 1960 *c*).

METHODS

Preparation of Oriented Nucleohistone for Electron Microscopy

Nucleohistone was prepared in the usual way with saline-Versene (Zubay and Doty, 1959). The final salt-washed sediment was stored in a stoppered container for 4 days at 5°C. During this interval of time the sediment becomes an elastic gel from which it is possible to pull a sheet of material about 100 microns thick by stretching the gel over two wooden rods and pulling these apart. The stretched sheet is held in tension so that the plane of the sheet is horizontal. Osmium fixative is applied dropwise so that the top surface is completely covered by a solution. This is continued for 2 hours. The chemically treated sheet is subsequently dehydrated by rinsing in ethanol water solutions: 70 per cent alcohol, 1 hour; 85 per cent alcohol, 1 hour; 90 per cent alcohol, 1 hour; 100 per cent alcohol, 1 hour. The tension is released only after the sheet has been in absolute alcohol for 1 hour. The fixed, dehydrated nucleohistone is cut into small strips suitable for embedding in gelatin capsules. The strips are selected by the sharpness of their extinction angle under a polarizing microscope. Invariably the best material lies along the edge of the original stretched nucleohistone sheet.

Preparation of Tissues for Electron Microscopy

Small pieces (~ 1 mm^3) of tissue were removed from young male rats immediately after the sacrifice of the animal. They were fixed for varying times (see Results section) in 2 per cent osmium tetroxide solution buffered with veronal acetate (Palade, 1952) either at pH 7.0 or at pH 6.0 in the presence of 10^{-2} M $CaCl_2$ (Kellenberger *et al.*, 1958). The temperature of the fixative was maintained at 0°C by immersion in melting ice. The tissue was dehydrated in an ethyl alcohol series (1 hour each in 70 per cent, 85 per cent, 90 per cent, 95 per cent, and 100 per cent, plus two further changes of 100 per cent ethyl alcohol, the tissue being left overnight in the last of these). For embedding in Araldite (Glauert and Glauert, 1958), the tissue was transferred to a 50-50 mixture of ethyl alcohol and the Araldite mixture without accelerator, for 2 to 3 hours; then into the Araldite mixture without accelerator, at 60°C for 2 to 3 days, and then into the final mixture for a further 2 days at room temperature, before transferring to capsules and allowing polymerization to take place in an oven at 60°C for 2 or 3 days. These times may seem unnecessarily prolonged, but they do eliminate troubles arising from incomplete infiltration of the plastic. Sections were cut with a glass knife, floated on a water surface, and collected on carbon-filmed Smethurst electron microscope grids. They were examined in a Siemens Elmiskop I, operated at 80 kv with the double condenser and a 50 μ objective aperture.

Staining of Sections for Electron Microscopy

Grids bearing the sections were immersed in the staining solutions for the requisite period of time. Staining with uranyl acetate was carried out at room temperature (20°C) using a 2 per cent aqueous solution whose pH was adjusted to 4.8 with dilute potassium hydroxide solution. The time of staining required for Araldite sections was usually 4 to 6 hours. Staining with lead hydroxide was carried out using a solution of the stain prepared in the manner described by Watson (1958*b*). In order to avoid contamination of the specimen by precipitated lead carbonate, the following procedure was adopted. The lead hydroxide solution was centrifuged at 20,000 *g* for 15 minutes with a layer of paraffin oil above it to protect its surface from the air; otherwise a surface precipitate forms and is constantly sedimented, leaving fresh surface exposed and allowing more precipitate to form. The clear solution was then syphoned off into bottles provided with special rubber stoppers; when a stopper was inserted into a filled bottle, any air bubbles were expelled through a hole in the stopper and through a short narrow tube leading from it. The tube was then closed off with a clip. Grids were stained without ever taking them directly through an air-liquid (staining solution) interface. This was done by first immersing them in a tiny glass cup containing recently boiled distilled water which was then transferred to the bottle of staining solution. After staining for about 1½ hours the grids were transferred, without being taken through an air-liquid interface, to a small U-tube, tapered on one arm so as to prevent the grids' being washed through, and about a hundred milliliters of boiled distilled water was passed through the tube. (The total washing time should not exceed 1-2 minutes.) The grids could then safely be dried. This procedure, though tedious, works very satisfactorily.

Feulgen Staining

Sections were transferred from the trough of the microtome to a glass slide by means of a platinum loop and allowed to dry. They were subjected to hydrolysis for 20 minutes at 60°C in 1 N HCl, and then transferred to the Feulgen solution (Gurr, London; pH adjusted to pH 2.2), where staining was allowed to proceed for 1 hour. They were then rinsed in the usual way and a permanent mount was made using Euparal (Flatters and Garnett Ltd., Manchester, England). Unhydrolysed controls gave negligible staining.

Use of Electron Microscope under High Resolution Conditions

Since structures extending down to the limit of resolution of the microscope (below 10 A) were often visible, it was worth while to set the astigmatism correction with high accuracy, and to focus to a similar degree of precision (better than 1 click on the fine focus control, equivalent to $\Delta f \sim 700$ A). This was done using carbon films with small holes in them as specimen supports, rechecking the astigmatism correction near the place on the grid which it was desired to photograph, and using the edge of a hole as a focusing aid. The perforated carbon films were prepared as follows. A clean glass slide was dipped into a 0.25 per cent solution of collodion in amyl acetate and allowed to drain in a vertical position. Just before the layer of liquid was completely dry, one gently breathed on the slide, so that on drying the surface appeared slightly cloudy. Immediate examination in the phase contrast microscope would reveal whether the holes so produced in the collodion film on the slide were of the required size range. The film was then stripped on a water surface, collected on an array of electron microscope grids, and dried. Carbon was then deposited in an evaporator and the collodion dissolved in butyl acetate.

RESULTS

Preliminary trials showed that fibres of nucleohistone, though well oriented both before and after fixation (as judged from their birefringence and the sharpness of the extinction they gave), showed only very poor orientation, and much evidence of polymerization damage, when embedded in methacrylate and sectioned. This loss of orientation was not experienced when Araldite (Glauert and Glauert, 1958) was used as an embedding medium. Accordingly, Araldite was employed throughout this work.

Much difficulty was also experienced at first owing to failure of the embedding medium to penetrate the oriented fibres adequately; it appeared that the oriented material had, during fixation and dehydration, become too densely packed to be infiltrated readily by the plastic. This effect could be avoided if 10^{-2} M $CaCl_2$ was included in the buffered osmium tetroxide fixative (pH 6.0). Kellenberger *et al.* (1958) also recommend the use of calcium in the fixation medium for bacterial DNA; but the medium we have used does not also involve the presence of amino acids. This modified fixative was used throughout this work, although its employment (as against a fixative lacking calcium) appeared to make little difference to the results in the case of whole tissue.

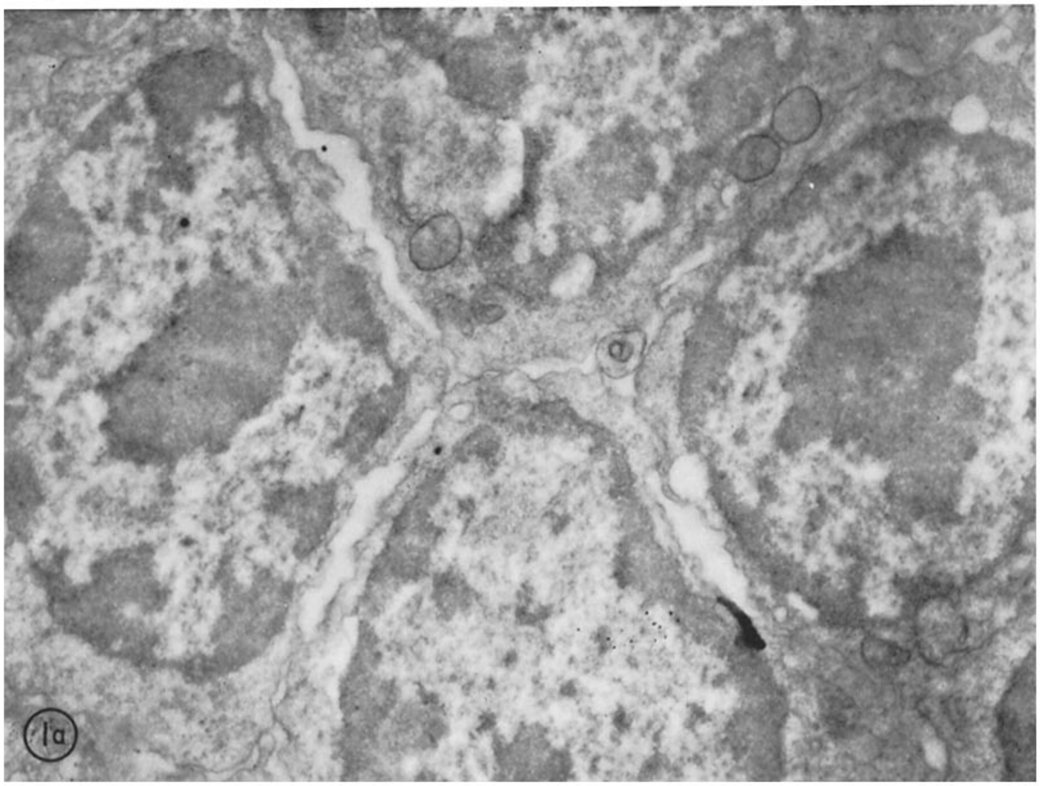
A number of heavy metal salts were selected in largely empirical fashion and made up in 2 per cent aqueous solution as possible staining agents for the embedded and sectioned material. The following compounds were tested: barium iodide, bismuth trichloride, lanthanum nitrate, lead chloride, lead acetate, mercuric acetate, mercuric nitrate, strontium bromide, thorium chloride, thorium nitrate, uranyl acetate, uranyl chloride, uranyl nitrate. Of these, uranyl acetate was found to produce by far the largest increase in contrast and to show up detailed and well oriented structure in the nucleohistone, and its action was investigated in some detail. Whilst our early work was in progress, other reports appeared in the literature concerning the employment of uranyl acetate in electron microscopy as a stabilizing agent (Kellenberger *et al.*, 1958) or as a stain (Watson, 1958*a*; Valentine, 1958). Valentine comments on the possibility that uranyl acetate may give specific staining of nucleic acid in adenovirus preparations, but Watson found, using tissue sections embedded in methacrylate, that uranyl acetate was incorporated with little specificity into most of the tissue components normally observed. Kellenberger *et al.*, working with phage-infected bacteria, found that their procedure, in which the material is treated with aqueous uranyl acetate immediately after fixation, produced an increase in contrast both of the phage and of the bacterial cytoplasm, and they commented that at least the cytoplasmic granules (ribonucleoprotein) were stained in addition to the phage. Watson (1958*b*) also reported that lead hydroxide strongly stains the RNA-containing particles in a variety of cells, but was cautious to point out that the affinity could be either for RNA, or for ribonucleoprotein, or for a particular protein. The results reported here show that by using somewhat different techniques the specificity of these stains can be substantially improved.

Staining of Tissue Sections

1. *Specificity of Uranyl Acetate Staining:* Having ascertained that nucleohistone could be satisfactorily preserved and stained in the manner described, we carried out some experiments on whole thymus tissue (from rat) to investigate the specificity of the staining. The tissue was fixed in the osmium-calcium medium and embedded in Araldite. It was found that sections stained on the grid in 2 per cent aqueous uranyl acetate for 4 to 6 hours showed a very large increase in the density of certain regions of the nucleus, and in many small particles of the cytoplasm, as compared with unstained controls. The effect is illustrated in pairs of successive sections in Fig. 1 *a* and *b*, the first member of the pair being untreated, the second stained with uranyl acetate. It is not possible to photograph a given area of the same section before and after staining, for exposure to the electron beam prevents subsequent uptake of stain. In the micrographs shown, the thickness of each section in the pair was as nearly as practicable the same, and the photographs were taken and enlarged

FIGURE 1

Rat thymus tissue, fixed in 1 per cent osmium tetroxide and embedded in Araldite. Successive sections. *a*, unstained; *b*, stained for 5 hours in 2 per cent aqueous uranyl acetate. The density of mitochondria and some other cytoplasmic structures is little changed by the staining, but the density of certain regions of the nuclei, and also of some cytoplasmic particles (presumably ribosomes), is greatly increased. Photographic conditions identical in the two cases. × 18,000.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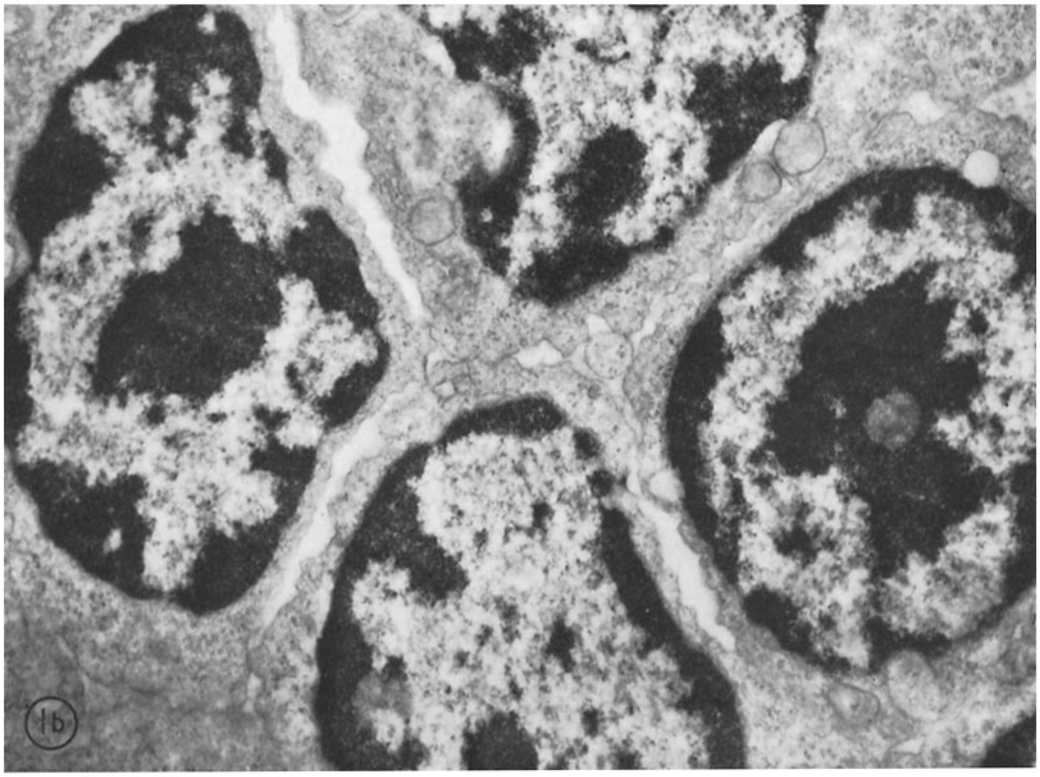
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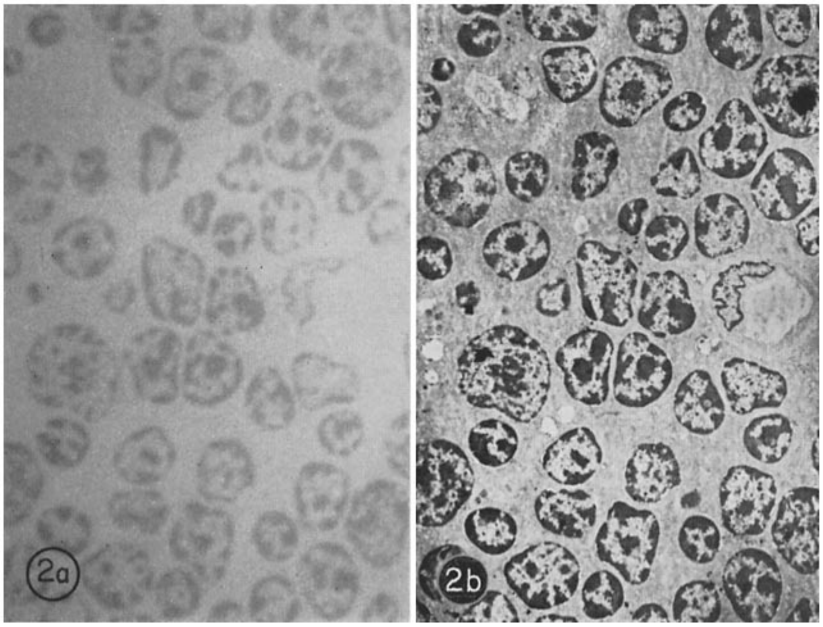


FIGURE 2

Rat thymus tissue, fixed and embedded in standard way. Successive sections. *a*, stained by Feulgen procedure and photographed in light microscope; *b*, stained in 2 per cent uranyl acetate for 5 hours and photographed in electron microscope. Note the very close correspondence between the regions of the nuclei stained in the two cases. × 2000.

under identical conditions. Thus the much greater increase in contrast after staining of certain regions of the nucleus as compared with other cell structures, *e.g.* mitochondria, cannot be due merely to a general increase in density, appearing more conspicuously in some structures by virtue of their higher initial density. The regions which stain strongly do not, initially, have a density which is singularly high as compared with other selected regions of the cell, and the density of many of these other regions, *e.g.* mitochondria, increases relatively little on exposure of the section to the uranyl acetate solution. A notable exception to this rule may be seen in the case of collagen fibres. Muscle filaments, on the other hand, are virtually unstained with uranyl acetate.

The densely staining regions of the nucleus seemed likely to be regions rich in nucleohistone; to investigate this, we have compared the distribution of uranyl acetate staining seen in electron micrographs with that of the classical Feulgen staining of DNA in adjacent sections examined in the light microscope.

2. *Comparison with Feulgen Preparations:* In preliminary experiments, it was gratifying to find that the Feulgen reaction worked very well on osmium-fixed tissue embedded in Araldite, without any special measures (such as removal of the plastic) being required. The sections were processed as described under Methods. Good staining of certain regions in the nucleus was observed, but we soon realized that accurate correspondence between these structures and those seen in the electron microscope in adjacent sections could only be looked for if the sections examined in the light microscope were almost as thin as the electron microscope sections. If sections 1 to 2 μ in thickness are used for the light microscope, the average density seen through this thickness in a given region can differ considerably from that sampled in an adjacent very thin section.

Fortunately, however, we found that sections only 0.25 μ to 0.35 μ were thick enough to examine with ease in the light microscope after Feulgen staining and gave reasonably good correspondence with electron microscope sections; and on further study, we found that sections down to about 1000 A in thickness (*i.e.*, giving silver or straw interference colours), and thus not much thicker than the sections used for electron microscopy at this stage of the work, could be employed. The tissue in such sections viewed in the light microscope shows up with only very low contrast to the eye, but perfectly adequate images were obtained on high contrast film (*e.g.* Kodak Microfile). Using such sections, a rather exact correspondence was found between the areas stained by the Feulgen reaction and those stained with uranyl actate. This is illustrated in Fig. 2 *a* and *b*. The Feulgen method is highly specific for DNA, so the results show that the regions in the nucleus whose density is greatly increased by treatment with uranyl acetate are those which contain substantial amounts of DNA.

To find out whether it is the DNA itself which is stained by uranyl acetate, or whether it is some other component associated with it, we have measured by direct weighing the uptake of stain by samples of purified DNA, of nucleohistone, and of histone, exposed to aqueous solutions of uranyl acetate of various concentrations. As might be expected from the great increase in contrast seen in the electron microscope, the increase in the dry weight of DNA-containing material is quite substantial. Purified DNA will take up an amount of stain almost equal to its own dry weight. Nucleohistone will take up about 50 per cent of its dry weight, and purified histone protein only about 20 per cent. These results are illustrated in Fig. 3. It is clear that a considerable part of the strong and preferential staining of nuclear regions in thymus tissue is due to the uptake of uranium by the DNA situated there. But of course it by no means necessarily follows that *all* the DNA originally present before fixation is still available for staining in the sectioned tissue.

The chemical nature of this uptake is not entirely clear. Quantitatively, it is equivalent to slightly less than one molecule of uranyl acetate per phosphate group. Other uranyl salts—*e.g.* chloride and nitrate—give weaker staining and much smaller uptakes than the acetate, so it does not appear to be just a simple binding of uranyl ions that is involved.

Uranyl acetate is a weak electrolyte in aqueous solution, indicating that the acetate ion is for the most part associated with the uranyl ion. In contrast, uranyl chloride and uranyl nitrate are strong electrolytes. This association of acetate with uranium and the correspondingly greater affinity of the uranyl acetate for nucleic acid–containing substances strongly suggests that the acetate plays a role in augmenting the extent of binding. It seems likely that this role of the acetate is related to a charge saturation phenomenon. First, consider the case of uranyl binding to DNA in aqueous solution. The affinity of UO_2^{++} for phosphate is well known. If the divalent uranyl ion alone were to bind to DNA, one would expect it to be absorbed strongly at first but with sharply decreasing affinity as over-all neutrality is reached. At neutrality there would be 0.5 UO_2^{++} ions per nucleic acid phosphorus, since each phosphorus atom carries a single negative charge which should result in a weight increase of the DNA of about 40 per cent. The observed maximum weight increase in the case of uranyl acetate binding to DNA is about 90 per cent, indicating that about one UO_2^{++} is bound per phosphorus. The most logical explanation of this high binding is that the UO_2^{++} carries with it some acetate, so that it is absorbed as the singly charged $UO_2(Ac)^+$ ion or the neutral $UO_2(Ac)_2$ species. This would allow the observed binding to take place without the build-up of a prohibitive concentration of positive charge on the surface of the DNA. An explanation of this type could also apply to the binding of uranyl acetate by nucleohistone and nucleoprotamine in aqueous

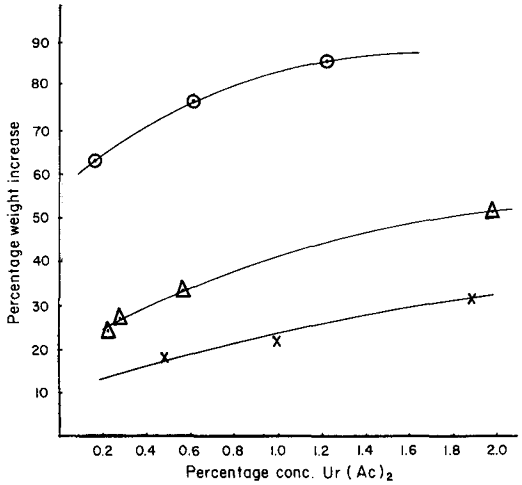
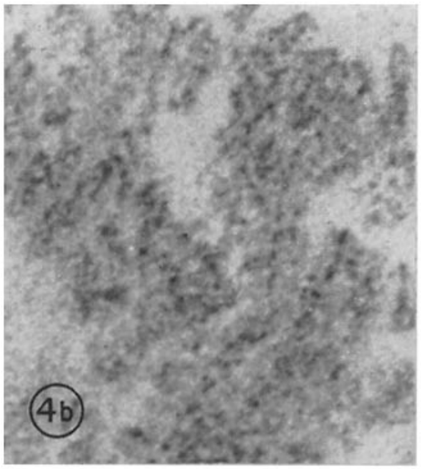


FIGURE 3

Uptake of uranyl acetate from aqueous solutions of various concentrations, expressed as a percentage of the original dry weight of the substance concerned. O—O—O, purified DNA; X—X—X, purified histone; Δ—Δ—Δ, nucleohistone.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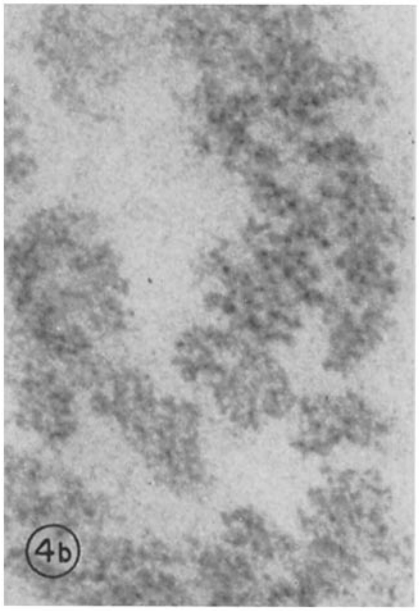
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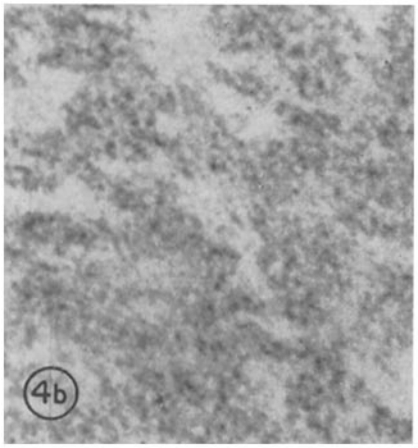
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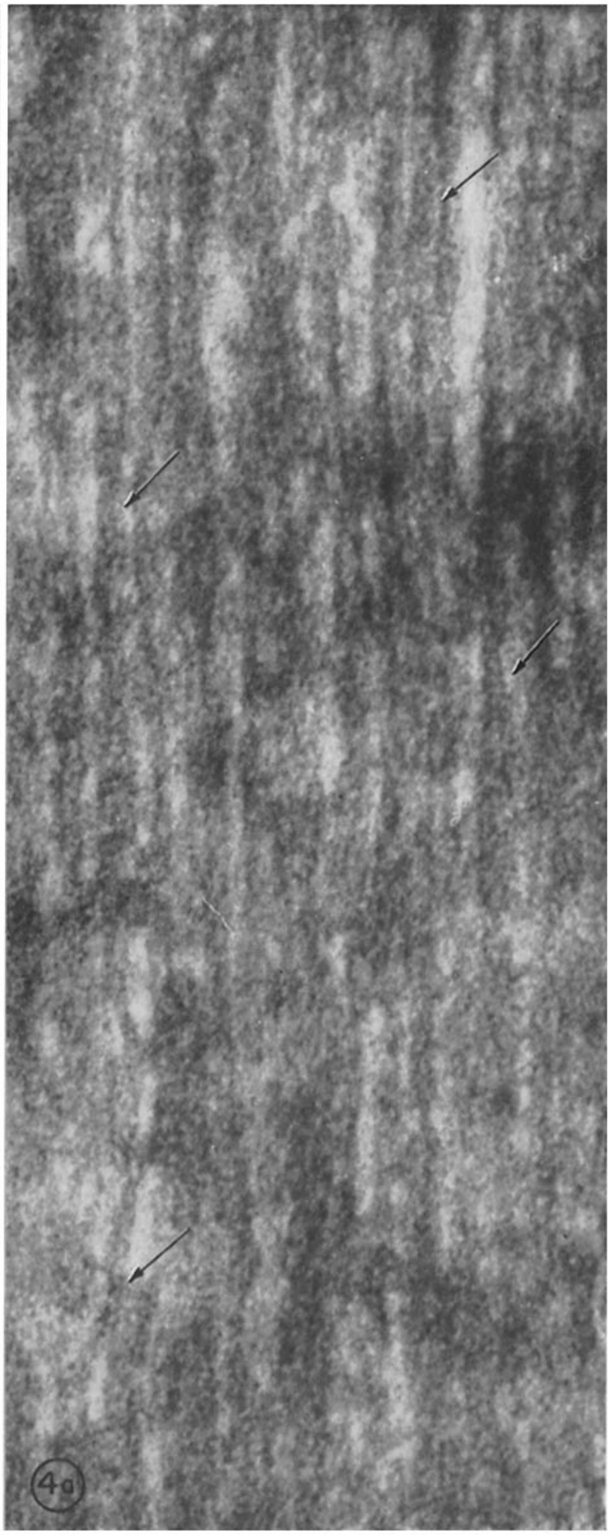
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solution. If the binding to these almost electrically neutral nucleoprotein complexes took place through the divalent uranyl ion, we would not expect it to be an additive function of the binding to highly negatively charged DNA and highly positively charged protein alone, as is in fact the case.

3. *Electron Microscope Observations on Nucleohistone:* Anticipating some of the results described later, it was found that the procedure which gave most intense staining was to employ uranyl acetate followed by lead hydroxide on the same section. The micrographs of sections of oriented nucleohistone discussed below were obtained by this method. The nucleohistone shows a highly characteristic appearance, but we have not so far been able to establish what is the underlying structure which gives rise to this appearance. In longitudinal sections (Fig. 4 *a*) the nucleohistone can be seen to be organized into well oriented, anastomosing bundles of variable diameter in the range of 50 to 200 A. These bundles themselves often show considerable internal structure, usually in the form of a meshwork or plaited appearance, the pores of the mesh having an axial spacing of about 60 A. This periodic structure sometimes appears simply as a series of dots, and sometimes as a short series of cross-connections between very thin longitudinal filaments. No doubt much of the difficulty in interpretation arises from the large number of superimposed structures which we are seeing within the thickness of even the thinnest sections (150 to 300 A). In transverse sections (Fig. 4 *b*) the material is present in irregular bundles of varying size, and within the bundles the end-on view of what may be cross-linked filaments can perhaps be discerned. The measured diameter of these filaments in the electron micrographs is 10 to 15 A and their minimum separation is 25 to 30 A. These values are of the general order that one would expect from single DNA or nucleohistone molecules, and it may well be that the micrographs are showing the DNA helices in end-on view. In side view (longitudinal sections) there would usually be six to twelve layers of such chains superimposed, and it would not be surprising if the resultant appearance was difficult to interpret, particularly if the chains were extensively cross-linked to one another. Thus, the preparative methods used here enable one to see a high degree of detail, down to the 10 A level, in sections of nucleohistone; this detail is oriented, characteristic, and consistent, and not in conflict with ideas on nucleohistone structure derived from x-ray diffraction studies (Wilkins *et al.*, 1959); further work is now needed.

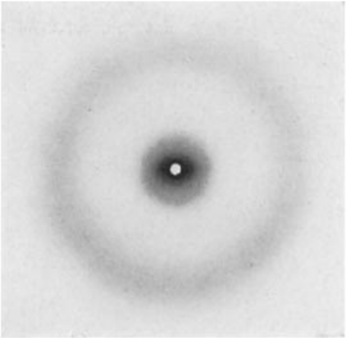


FIGURE 5

X-ray diffraction diagram from oriented fibre of extracted nucleohistone fixed and embedded in standard way. Four diffraction rings are present, corresponding to spacings of 4.1 A, 18 A, 37 A, and 66 A. The 4.1 and 18 A diffraction rings arise from the Araldite embedding medium, and should be discounted. The 37 A and 66 A diffraction rings arise from the nucleohistone, and this is the characteristic pattern obtained from fully hydrated fibers of unfixed nucleohistone. Copper Kα radiation; specimen film distance 5.1 cm.

We have also obtained x-ray diffraction diagrams (Fig. 5) from fixed specimens of oriented nucleohistone embedded in Araldite, which resemble closely those obtainable from unfixed fibres

FIGURE 4

Oriented fibre of extracted nucleohistone, fixed and embedded in standard way; sections stained with 2 per cent aqueous uranyl acetate for 6 hours followed by lead hydroxide staining for 1½ hours. *a*, longitudinal section, × 300,000; *b*, cross-sections, × 500,000. Regions in the longitudinal section showing the characteristic fenestrated or plaited appearance indicated by arrows. In the cross-sections, the separation of the dense dots (which may represent rod-like structures in end-on view) is 20 to 30 A.

of oriented nucleohistone in the fully hydrated state, showing for instance the characteristic 37 A and 66 A diffraction rings. These observations again suggest that the preparative procedures are preserving a good deal of the original organization of the nucleohistone, at least when it is already oriented into fibres.

We now return to the observations of staining of intact tissue.

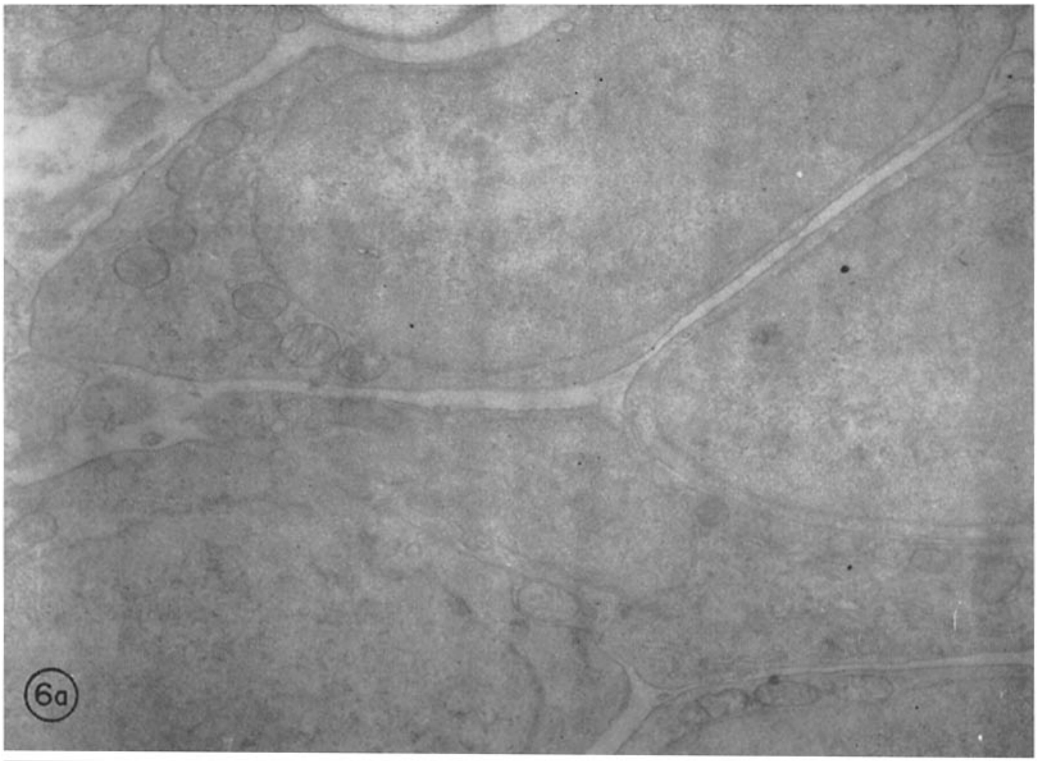
4. *Staining of Whole Tissue with Lead Hydroxide:* We have investigated the specificity of the lead hydroxide staining method (Watson, 1958b) on Araldite-embedded tissue, particularly rat thymus. In material fixed for ½ hour at 0°C, the general pattern of staining was found to resemble closely that obtained with uranyl acetate, although the intensity of staining is often greater with lead hydroxide. There is strongly preferential uptake of stain by the nucleic acid–containing regions of the cell, *i.e.*, certain regions in the nucleus and the microsomal particles in the cytoplasm. This is illustrated using pairs of stained and unstained sections in Fig. 6 *a* and *b*. However, the relative extent of the staining of the DNA- and RNA-containing regions appears to depend on fixation time; this effect will be described in a later paragraph.

5. *Effect of Fixation Time on Subsequent Staining of Tissue Sections with Uranyl Acetate:* In the earlier part of the work a fixation time of ½ hour was generally used. Specimens were inspected during the course of dehydration to confirm that they were fixed (as judged by the extent of blackening) throughout their depth, which was usually substantially less than one millimetre. However, although all sections showed the same general pattern of staining by uranyl acetate, some seemed to stain much more intensely than others; this effect was not due to variation in section thickness or to variations in the hardness of the Araldite. The responsible factor was eventually found to be the duration of fixation. Lightly fixed tissue blocks carefully sectioned through their original outer boundaries showed a clearly marked gradient in the appearance of the cells from the outside surface towards the centre of the block. In blocks fixed for 15 minutes (at 0°C), the outermost layer of cells, though well preserved, stained much less strongly with uranyl acetate than the layer immediately below, while farther from the surface still, although staining was still strong, fixation was clearly inadequate. The whole range of effects could be observed within a depth of a few hundred microns or less. Sections from blocks fixed for 5 minutes showed, after uranyl acetate treatment, a narrower, weakly stained peripheral zone and still contained a useful layer of well fixed and strongly stained cells. The appearance of cells in such an area of rat pancreas is shown in Fig. 7; with more conventional fixation times, the contrast of the nuclei is much reduced. Even blocks fixed for only 2 minutes showed some useful areas. We have adopted the 5-minute fixation time as standard procedure, taking care, of course, to reject those parts of the specimen which, from their colour, are obviously unfixed.

6. *Effect of Fixation Time on Staining by Lead Hydroxide:* When sections of tissue (for instance rat thymus or pancreas) cut from blocks which were fixed for 5 or 15 minutes are stained in lead hydroxide and washed as we have described above, most regions of the nuclei are so weakly stained that the nuclei appear *paler* than the surrounding cytoplasm. The RNA-containing particles in the cytoplasm still stain moderately well, however, and in nuclei where a nucleolus is present in the section, this often stains quite strongly too. This can be seen in the section of rat thymus shown in Fig. 8. The main masses of chromatin in the nucleus are relatively unstained, but some of the material lying between these areas is quite strongly stained. Nucleoli are known to contain RNA and to be Feulgen-negative, *i.e.* presumably to contain little DNA. Serial sections showing the same areas of pancreas tissue stained with lead hydroxide and with uranyl acetate may be compared in Fig. 9 *a*

FIGURE 6

Rat thymus tissue, fixed and embedded in standard way (½-hour fixation). Successive sections (thinner than those in Fig. 1). *a*, unstained; *b*, stained with aqueous lead hydroxide for 1½ hours. Photographic conditions identical. There appears to be relatively little increase in contrast of the mitochondria, but, as in the case of uranyl acetate staining, certain regions of the nuclei, and the microsomal particles in the cytoplasm, stain strongly. × 17,000.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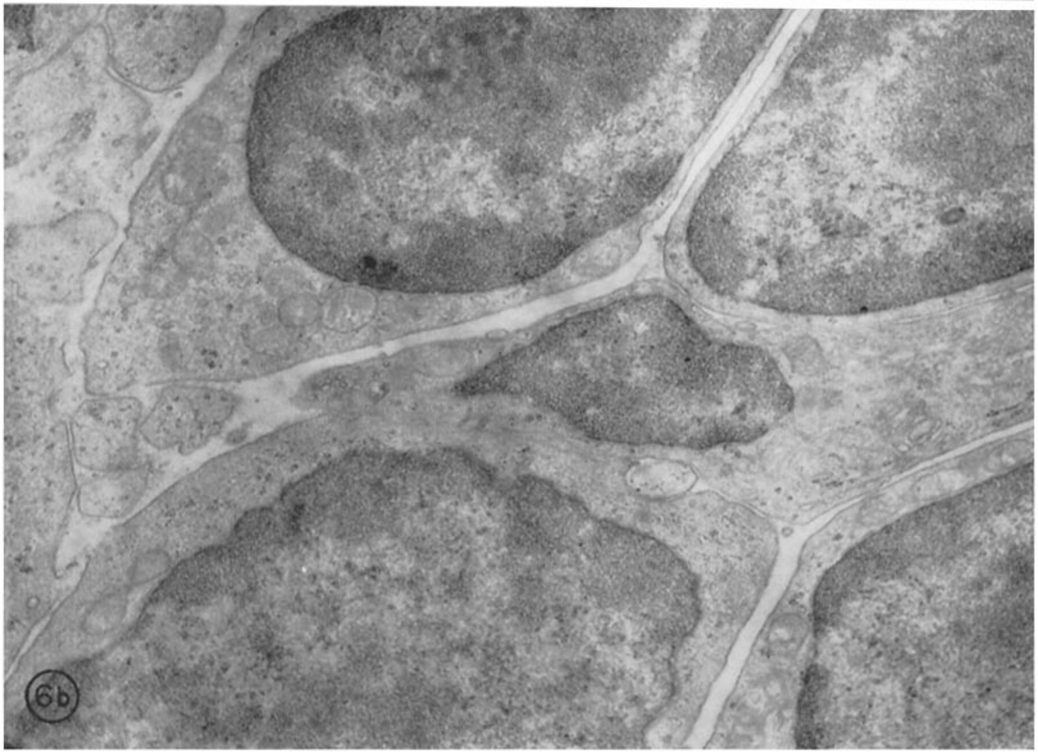

6b

and *b*. The nucleolus, stained in each case, is surrounded by nuclear material which stains strongly with uranyl acetate, and weakly or not at all with lead hydroxide; the staining of the cytoplasmic particles is similar to that of the nucleolus in the two cases. The appearance of the three types of structure—microsomal particles, chromatin, and nucleolus—after lead hydroxide staining of briefly fixed tissue is shown at higher magnification in Fig. 10. It should be emphasised that this effect is only obtained with short fixation times and that the zone of the tissue in which good nucleolar staining occurs is a narrow one. As the fixation time is extended, staining of the other regions of the nuclei with lead hydroxide is increased, whilst staining with uranyl acetate is decreased, so that with conventional fixation times the general pattern of staining in the two cases is very similar.

7. *Staining with Uranyl Acetate Followed by Lead Hydroxide:* If 5-minute-fixed tissue which has been stained in the section with uranyl acetate is subsequently exposed to lead hydroxide staining, a further large and preferential increase in the density of all the nucleic acid–containing regions takes place. Serial sections of rat thymus showing this effect are illustrated in Fig. 11 *a* and *b*. It seems that pretreatment with uranyl acetate renders the DNA-containing regions of lightly fixed tissue accessible to lead hydroxide staining.

If lead hydroxide staining is applied to tissue which has been fixed for ½ hour and stained in the section with uranyl acetate, again all the nucleic acid–containing regions of the cells increase in density; but the final density in this case is no greater, either in the cytoplasm or in the nucleus, than that obtained on the same samples with lead hydroxide alone. The staining in this case is weaker than that obtained by the uranyl-lead treatment of lightly fixed tissue.

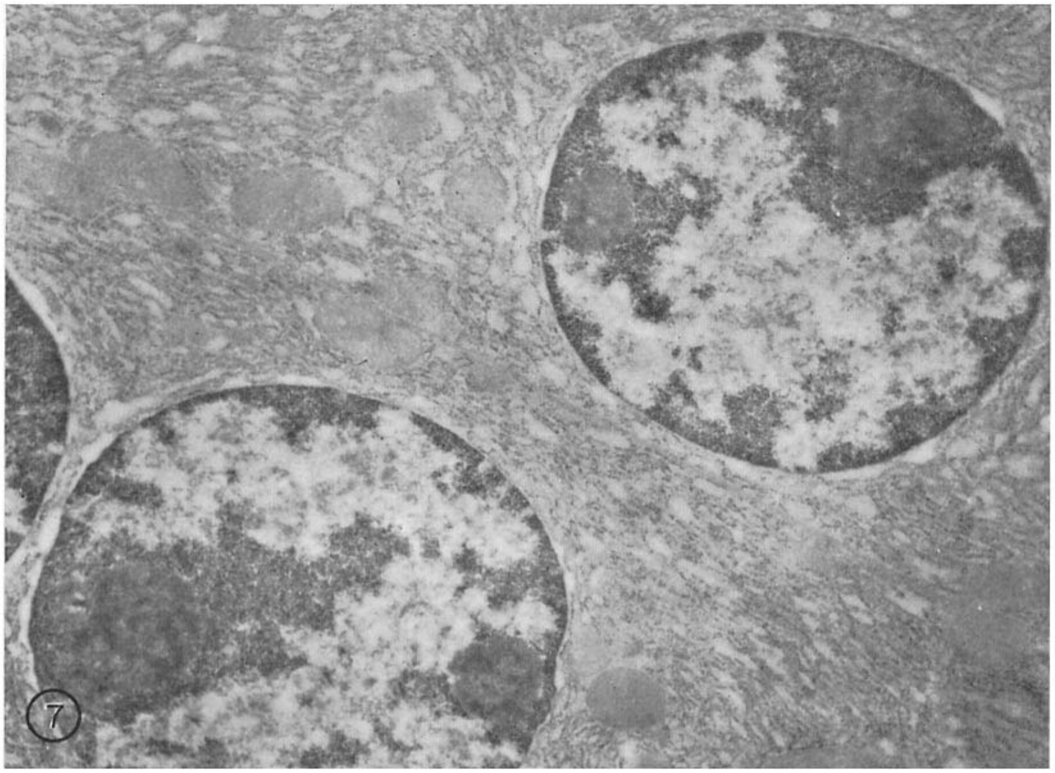
8. *Effect of Methacrylate Embedding on Staining of Tissues with Uranyl Acetate and with Lead Hydroxide:* We have been puzzled that the strongly preferential staining of nuclear regions by uranyl acetate and lead hydroxide has not already been the subject of more widespread comment, for under the conditions used here it is a very striking effect indeed (see, for instance, Fig. 1). The use of saturated aqueous uranyl acetate, as opposed to the 2 per cent solution that we have employed, does not make any noticeable difference in the pattern of staining produced. However, the use of methacrylate as an embedding medium does lead to substantial differences in the visible pattern of staining; a differential increase in contrast, in for instance thymus cells, between nuclear regions and mitochondria is very much less marked than when the same tissue is embedded in Araldite. It is rather difficult to compare the degree of nuclear staining in sections from two different blocks embedded in different plastics, but our impression is that it is less in the methacrylate-embedded tissue. On the other hand, the other structures in the cell show up with much better contrast in methacrylate even before staining—the poor contrast frequently provided by Araldite embedding being well known—so that any increase in density of the nuclear structures will stand out less obviously against the general background of the rest of the tissue than in Araldite; it is also possible that the accessibility of different cell components to the stain is affected unequally by the nature of the plastic in which they are embedded. Furthermore, the most intense staining with uranyl acetate is obtained using tissue fixed for much shorter times

FIGURE 7

Rat pancreas tissue, fixed for 5 minutes at 0°C, embedded in Araldite. Stained for 6 hours with 2 per cent aqueous uranyl acetate. The nuclei are well stained and stand out with good contrast above the cytoplasm. × 15,000.

FIGURE 8

Rat thymus tissue, fixed for 5 minutes at 0°C, embedded in Araldite. Stained for 1½ hours with lead hydroxide solution. There is some staining of particles in the cytoplasm, and of certain regions of the nucleus, notably those lying between the main masses of chromatin, and possibly of the nucleolus. But the main areas of the nucleus which characteristically stain with uranyl acetate and with the Feulgen reagent (see Figs. 1 and 2) are relatively unstained. × 19,000.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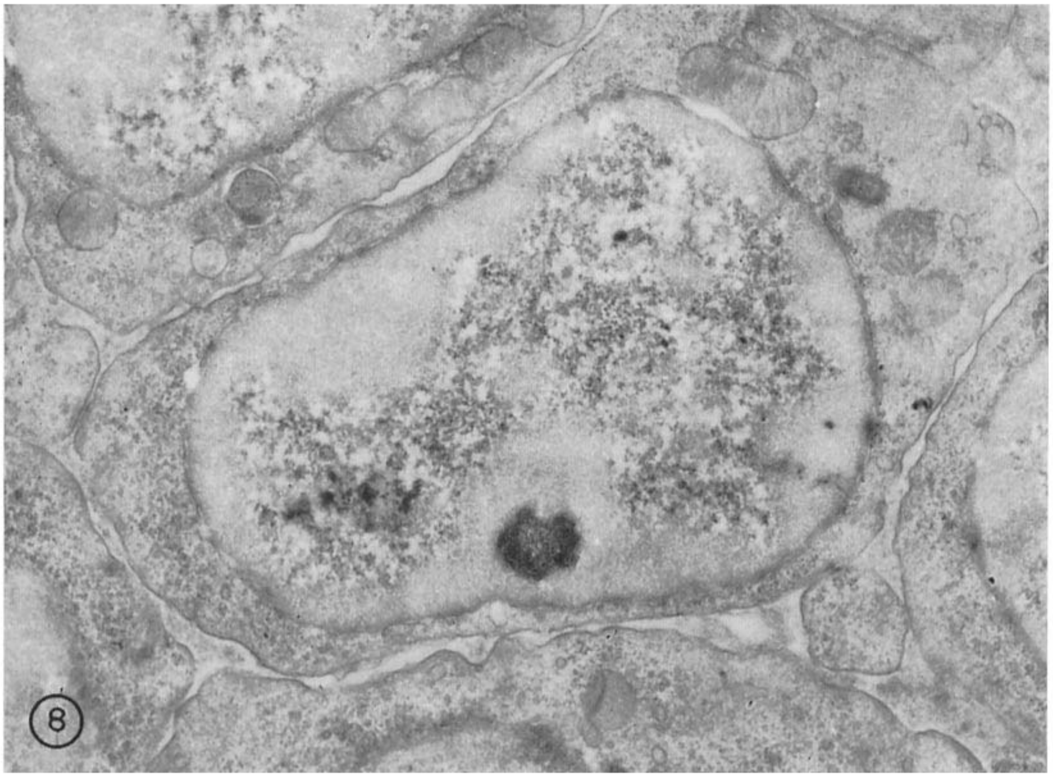
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than seem to be general practice. It is perhaps this combination of factors which results in the much stronger preferential staining obtained under the conditions of our experiments. Tissue embedded in Epon behaves similarly, in our limited experience, to that embedded in Araldite, except that the extent of differential staining appears to be somewhat less.

Staining of Other Nucleic Acid–Containing Structures

The apparent success of these efforts to stain nucleic acids in nucleohistone and in tissue sections encouraged the application of similar staining methods to a variety of other nucleic acid–containing structures, with a view both to ascertaining the pattern of specificity in other cases, and to finding out whether significant fine structure could be revealed.

The staining of ribonucleoprotein particles in the cytoplasm has already been mentioned, and we have published elsewhere (Huxley and Zubay, 1960*a*) the results of studies on analogous particles—ribosomes isolated from *E. coli*—by a combination of uranyl acetate staining and the negative staining technique (Hall, 1955; Huxley, 1956; Brenner and Horne, 1959). These methods have also been applied to a number of the small spherical plant viruses, containing RNA, and to phage particles, containing DNA.

Observations on Bushy Stunt Virus (BSV) and Turnip Yellow Mosaic Virus (TYMV): Bushy Stunt Virus is a small "spherical" plant virus with a molecular weight of about 10 million and a diameter of about 300 A. It contains approximately 14 per cent of RNA (Markham, 1951). For electron microscopy, specimens were prepared in the following manner. The virus particles were suspended by dissolving small crystals of virus in a few drops of distilled water. A drop of this suspension was then placed on a carbon-filmed grid, which was immediately rinsed with distilled water; if the concentration of the virus suspension is suitably chosen, large numbers of virus particles adhere firmly to the carbon film, often in the form of small regular arrays, usually with hexagonal close packing. The preparations may then be stained by immersing the grids in the staining solution, the particles remaining attached during this process and the subsequent rinsing.

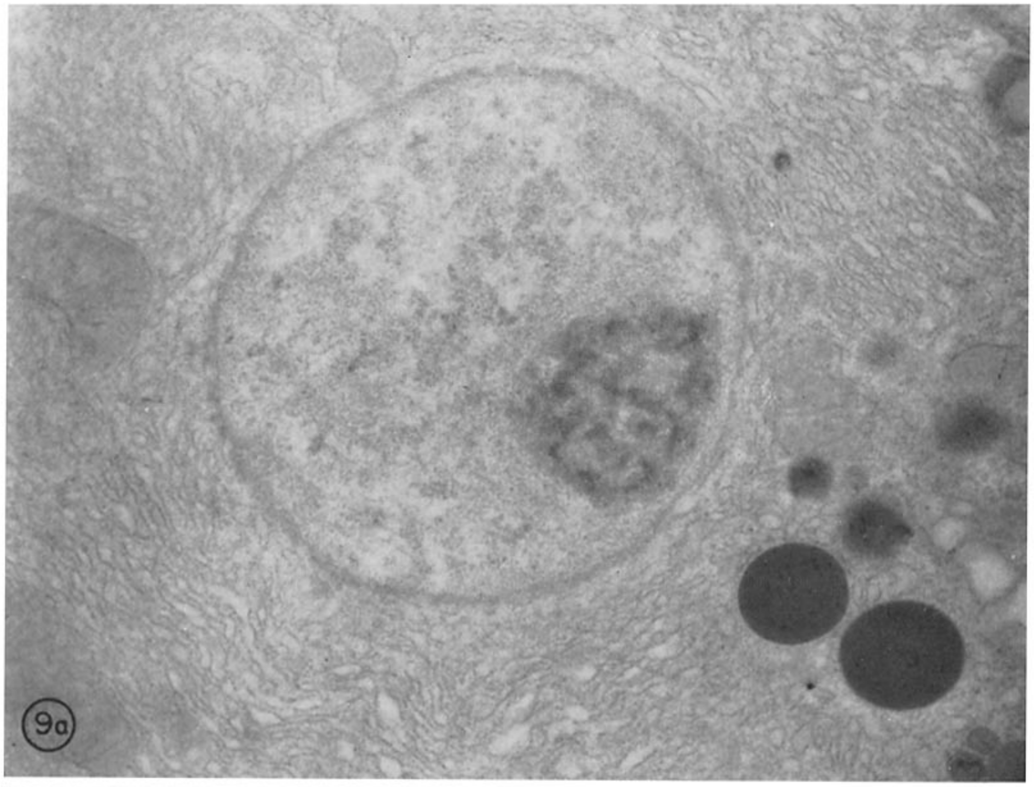
Staining with uranyl acetate can also be carried out by simply mixing a drop of suspension of virus in distilled water with one drop of 2 per cent or 4 per cent aqueous uranyl acetate, the preparation being kept in a moist chamber to prevent drying. Carbon-filmed grids can then be dipped in the preparation, rinsed, and dried, when they will be covered with stained virus particles.

The general appearance of such preparations is shown in Fig. 12. Arrays of roughly circular, densely stained areas are visible, having a diameter of about 180 A, but these do not lie in contact with their neighbours. The space between them seems to contain material of lower density, different, however, from the background density. We suspected that a core of the virus particles might be staining strongly, surrounded by a shell of weakly stained or unstained material, and that these outer shells were in contact with one another. This interpretation was confirmed by combining positive staining with uranyl acetate with negative staining, the external boundary of the particles being shown up by allowing uranyl acetate solution to dry down in the form of a thin film on the preparation. The appearance of this type of preparation is shown in Fig. 13. It will be seen that each particle consists of a stained core enclosed in a virtually unstained annular shell, whose outer boundary is clearly delineated by the external stain.

Preparations of Turnip Yellow Mosaic Virus treated with uranyl acetate also show an array of densely staining cores separated by relatively un-

FIGURE 9

Rat pancreas tissue, fixed for 15 minutes at 0°C, embedded in Araldite. Successive sections. *a*, stained with lead hydroxide ($1\frac{1}{2}$ hours); *b*, stained with uranyl acetate (6 hours). Staining of microsomal particles, and of the nucleolus, occurs in each case, but the mass of chromatin surrounding the nucleolus, and other areas elsewhere in the nucleus, while staining with uranyl acetate, are relatively unstained by lead hydroxide. × 15,500.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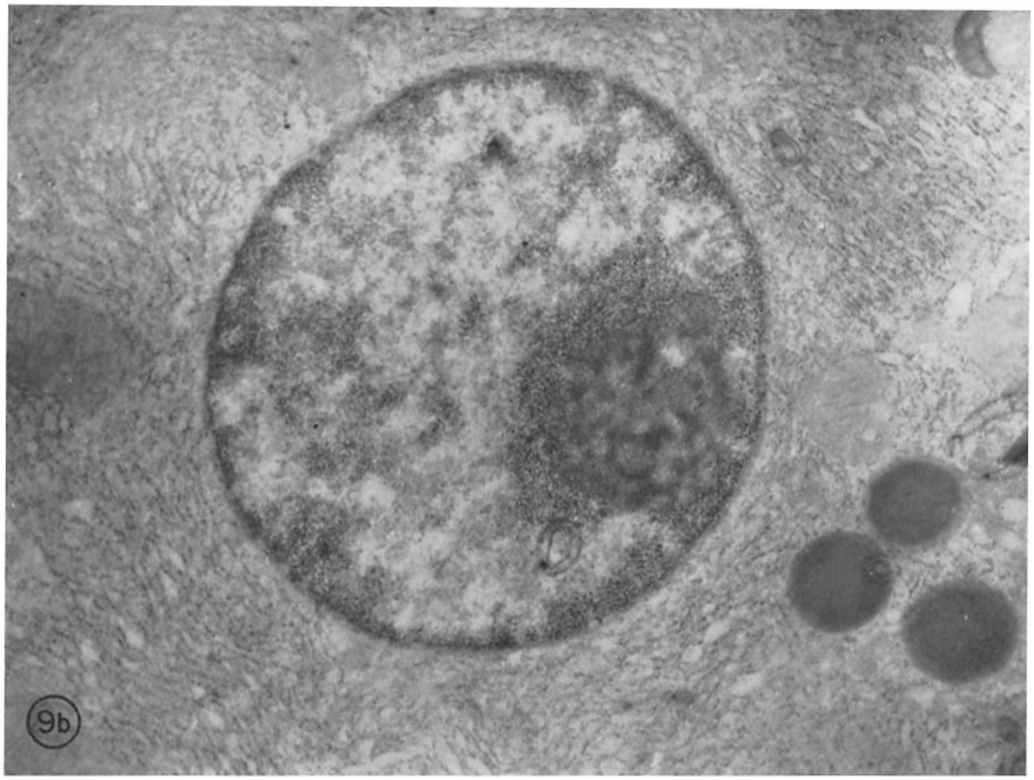
9b

stained peripheral regions (Fig. 14). There is very strong evidence from hydrodynamic and x-ray diffraction studies (Markham, 1951; Schmidt *et al.*, 1954; Klug *et al.*, 1957) that these virus particles consist of a central core of RNA and an external shell of protein, and, in fact, preparations can be obtained consisting largely of protein alone, without the RNA, but having the same size and external shape as the intact particles. When examined in the electron microscope, the preparations are found to consist very largely of empty shells (a few of which are always found in normal preparations of virus), which do not stain appreciably with uranyl acetate.

Thus it appears that in these two viruses, uranyl acetate is acting as a preferential stain for the nucleic acid core.

Apparent Fine Structure in Stained BSV: The central core of BSV stained with uranyl acetate often appears hexagonal in profile, as seen in Fig. 15. There is evidence (Kaesberg, 1956) that the virus as a whole has an icosahedral form. This figure would show a hexagonal profile when viewed in certain directions; such profiles are visible in negatively stained preparations of BSV (Huxley and Zubay, unpublished observations). Indeed, this hexagonal outline of the exterior of the particle can sometimes be discerned even in the uranyl acetate–stained preparations, particularly if they have been lightly fixed in osmium tetroxide beforehand. The hexagonal outline is oriented in the same sense as the core. It seems likely that the central core of the particles has an icosahedral form, matching the external shell.

The central core is not stained uniformly dense, but shows a great deal of internal fine structure (Fig. 16). The visible detail extends to the limit of resolution of the microscope (better than 10 A). Structure can very often be seen to better advantage in the original negatives than in the normal photographic enlargements from them—the contrast conditions in the former case seem more favourable to the eye—and so we have reproduced some of the micrographs as "reverse prints," made by direct contact printing from the normal enlargements (Fig. 16 *f* and *g*). The detail seen here reproduces accurately that visible in the original negatives. Examination of the positive enlargement in which the background detail is visible shows that the detail seen in the particles is not due to spurious structure also present in the supporting film seen between the particles. However, we hesitate to conclude that the structures seen in the electron microscope images give us a direct picture of the arrangement of the RNA in the virus. Although there is a certain general similarity in the appearance of all the particles, the details seem to vary from one particle to another more than one would expect merely as a result of viewing a constant structure from different directions. It seems therefore that more complicated factors determine the observed structural details.

The molecular weight of the RNA in the virus is about 1.4×10^6. A double-helical RNA chain of this size would have a length of some 7000 A. If a molecule or molecules of this total length are contained within a core whose diameter is only about 180 A, then there will be inevitably a great deal of overlapping in the projected view of this seen in the electron microscope. The visibility of

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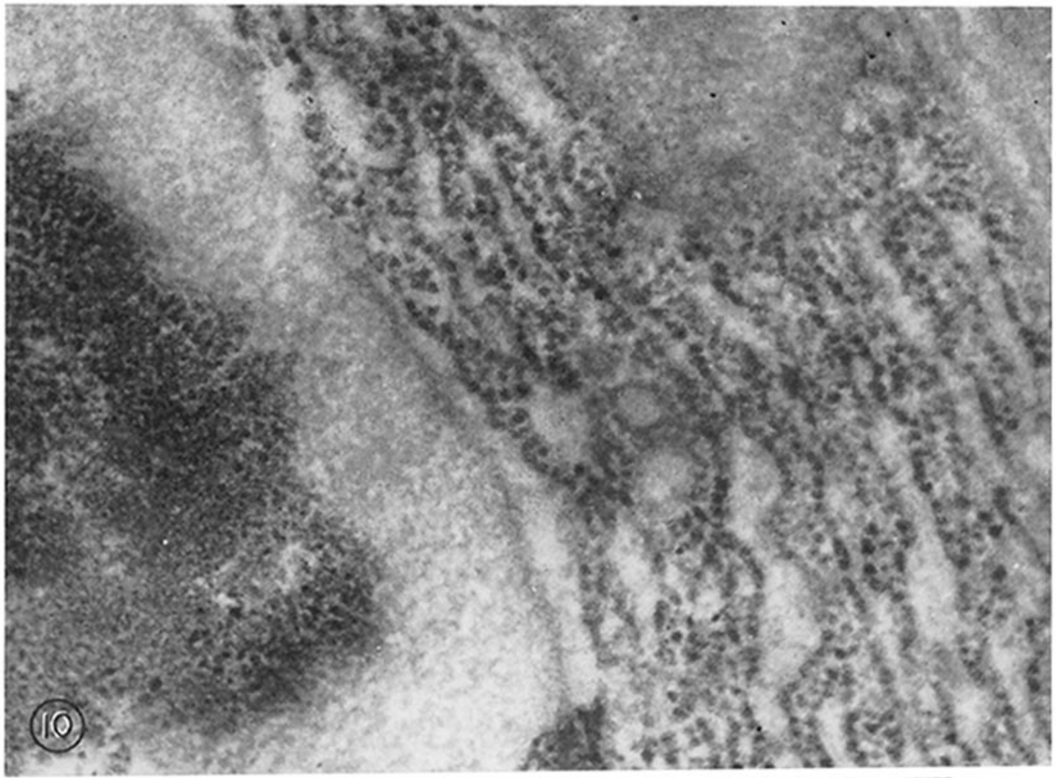
FIGURE 10

Rat pancreas tissue, 5-minute fixation at 0°C, embedded in Araldite, stained $1\frac{1}{2}$ hours with lead hydroxide. The strong staining of microsomal particles and of the nucleolus, and the weak staining of the chromatin between the nucleolus and the nuclear membrane, are well marked. × 80,000.

FIGURE 11

Rat thymus tissue, 5 minutes fixation at 0°C, Araldite embedding. Successive sections. *a*, stained for 6 hours with 2 per cent aqueous uranyl acetate; *b*, stained as in *a* and then further stained for $1\frac{1}{2}$ hours with lead hydroxide. Photographic conditions identical. Although the nuclei of tissue fixed and embedded in this manner were virtually unstained by lead hydroxide alone, a large increase in density of the areas already stained by uranyl acetate was obtained on subsequent staining with lead hydroxide. × 20,000.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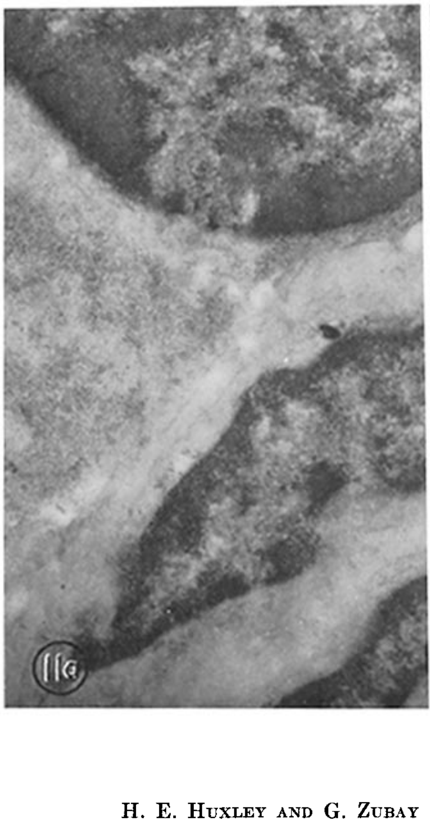
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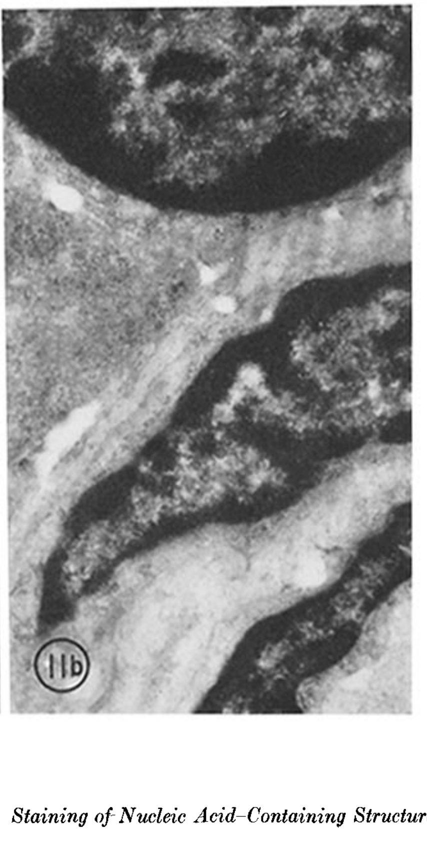
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Figure 12

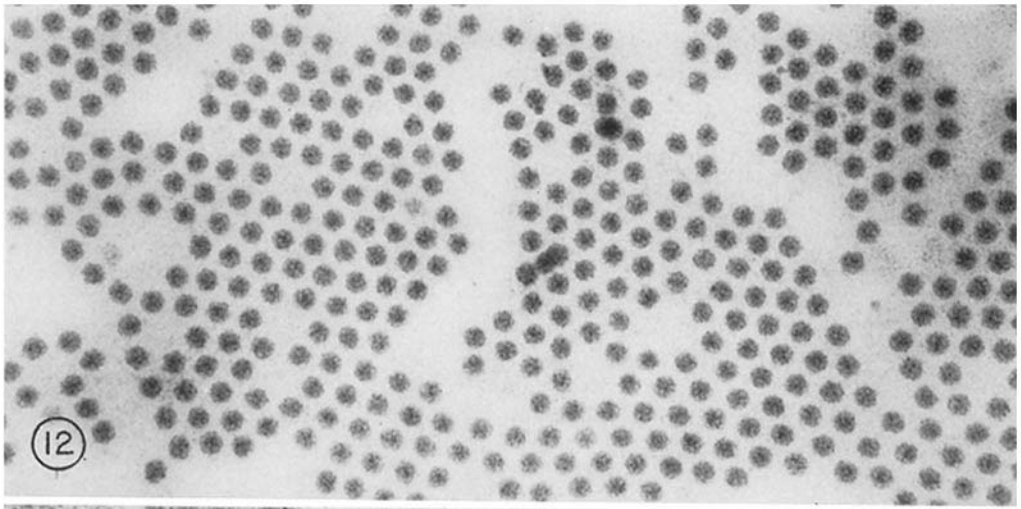
Bushy Stunt Virus (BSV), adhering to carbon supporting film and stained for 6 hours with 2 per cent aqueous uranyl acetate. The regions of the particles which take up the stain are separated from neighbours by an unstained region which it is believed represents the protein shell of the virus particles, which are in contact with one another. The stained region seen here would represent the nucleic acid core. × 150,000.

Figure 13

BSV, as in Fig. 12, but with thin layer of uranyl acetate solution allowed to dry down on particles after staining. The particles are outlined by the external uranyl acetate, and the relatively unstained shell between this and the stained core can now be seen. × 150,000.

Figure 14

Turnip Yellow Mosaic Virus (TYMV), adhering to supporting film and stained for 6 hours with 2 per cent aqueous uranyl acetate. This virus too appears to have a densely staining core, surrounded by a more lightly stained outer shell. × 150,000.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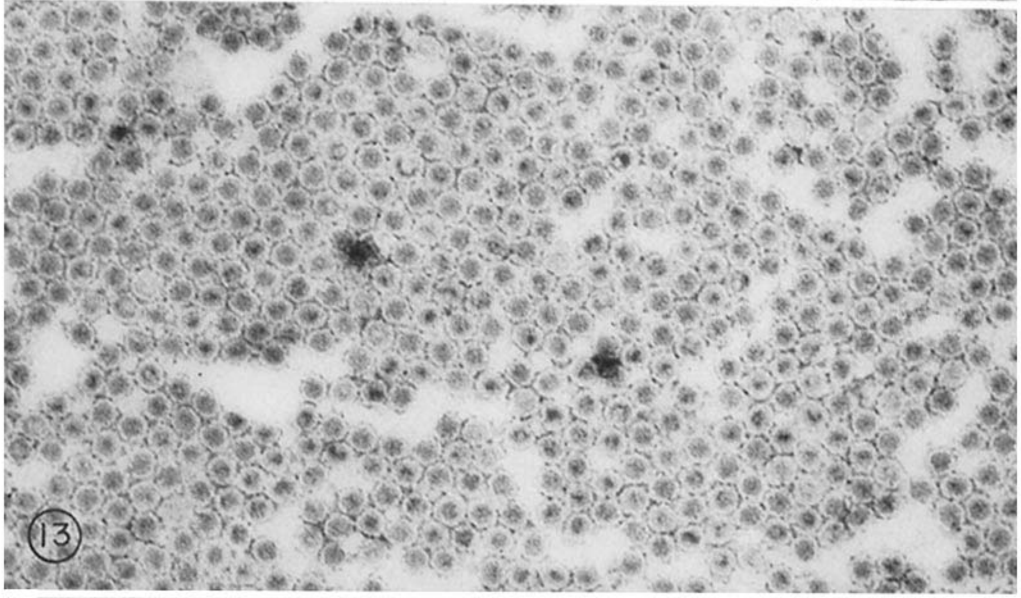
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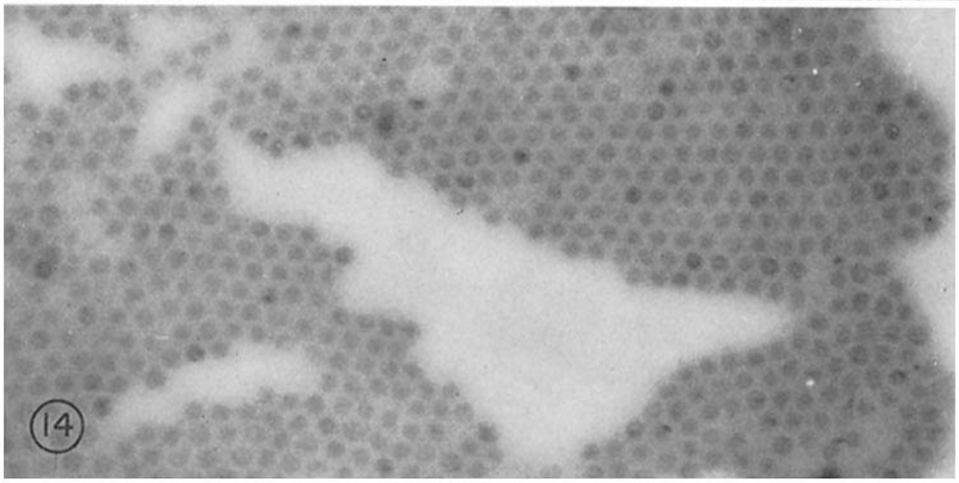

14

certain regions may increase quite sharply when they are in exact register with others at a different depth, and the appearance of the structure in micrographs may therefore depend very critically on the exact orientation of the particles. Again, it is possible, indeed likely, that the uranium is bound to the RNA at specific sites arranged with a regular spacing along the chain molecules. For certain orientations where the Bragg reflection conditions were satisfied, stronger scattering of electrons would occur, and so chains in these particular orientations might show up with exaggerated contrast. Finally, it is quite possible that the RNA structure becomes somewhat disordered by the preparative treatment, particularly when it is dried, and that the extent and nature of the disorder varies somewhat from one particle to the next.

Bearing all these reservations in mind, we would like to draw attention to some persistently recurring features of the electron microscope images of the stained cores of BSV. A substantial proportion of particles show small fenestrations, often polygonal in outline, having a diameter of 30 to 40 A and often occurring in small groups to form a honeycomb-like appearance. Occasional particles (*e.g.* Fig. 16 *g*) show a striking degree of regularity in the arrangement of these fenestrations, but these are exceptional. The width of the densely staining lines bounding the fenestrations is of the order of 10 A. The pattern of fenestrations is too variable at present to be described in terms of any particular regular polygon seen in projection. One may note, however, that a regular polygon with a radius of 80 to 90 A and faces 35 A across would have about 80 to 90 such faces, and that the total length of the edges would be about 4000 A. There is thus enough RNA present to build almost two layers in such a polygonal meshwork, even if the RNA were double-helical throughout. It is interesting that somewhat similar fenestrations were seen also in preparations of purified ribosomes stained with uranyl acetate (Huxley and Zubay, 1960*a*).

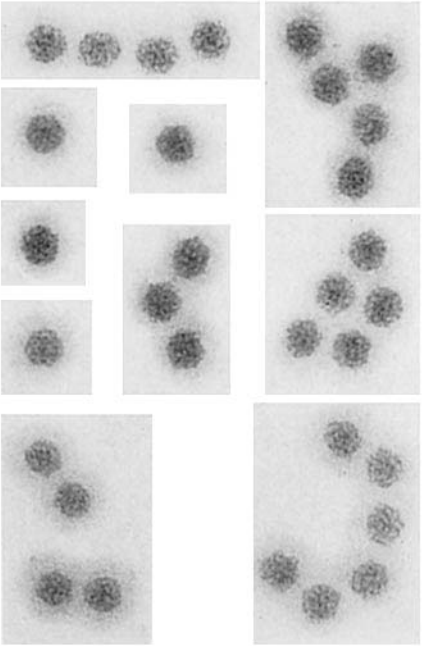


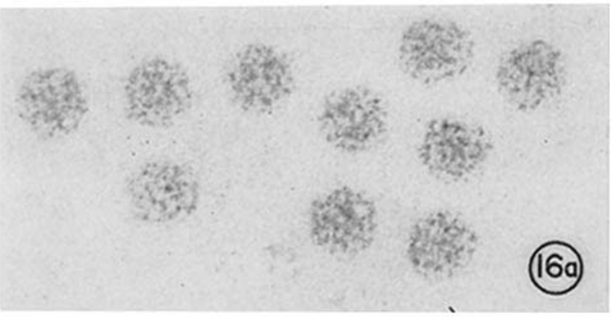
FIGURE 15

BSV, stained with uranyl acetate, showing the characteristically hexagonal profile of the stained core of many of the virus particles. In some cases, the external shell also appears hexagonal in profile. × 300,000.

Staining of Virus Particles and Phage with Lead Hydroxide: Untreated virus and phage particles do not stain with lead hydroxide. However, if the particles are first stained with uranyl acetate, then a further increase in density is produced by lead hydroxide. This is particularly well marked in the case of phage (T2) which contains DNA. The phage particles are too densely packed with DNA to show internal detail in unsectioned preparations (though some indication of detail is visible in thin sections), but the large uptake of stain into the head of the phage is quite striking and is illustrated in Fig. 18, which also shows a T7 phage. The failure of the particles to stain with lead hydroxide unless pretreated with uranyl acetate (or,

FIGURE 16

BSV, stained with uranyl acetate, showing in the cores considerable fine structure of debatable significance (see text), often having a fenestrated appearance. The particle indicated by the arrow, in the pair of positive and negative prints (*f* and *g*), is an exceptional one, but particles showing some evidence of fenestration are found in all preparations. *a* to *e*, × 500,000; *f* and *g*, × 400,000.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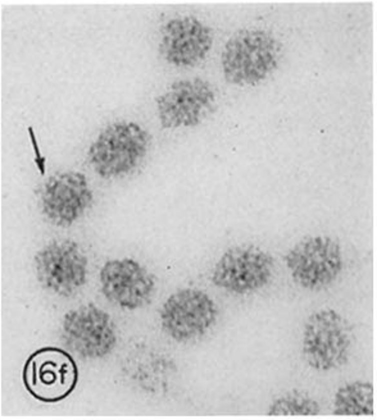
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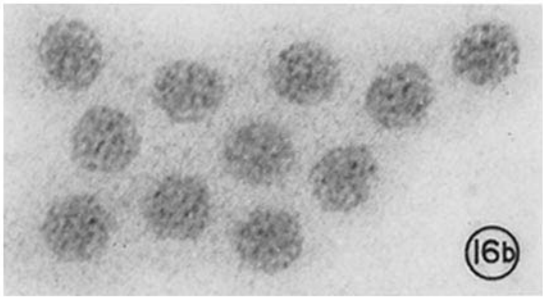
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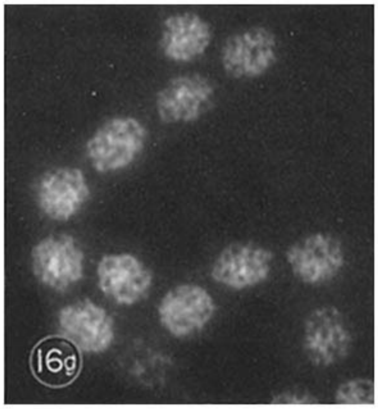
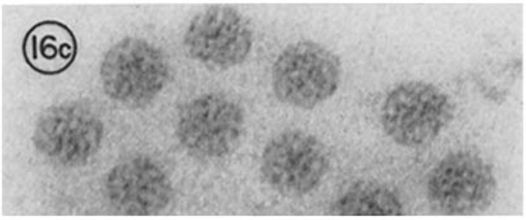

16g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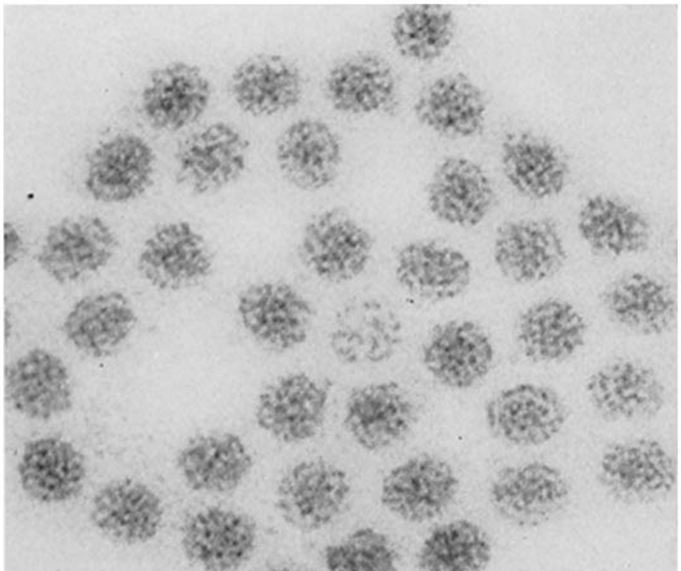
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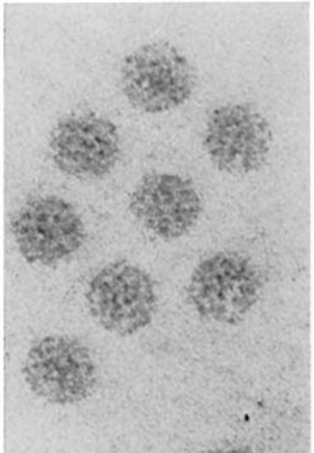
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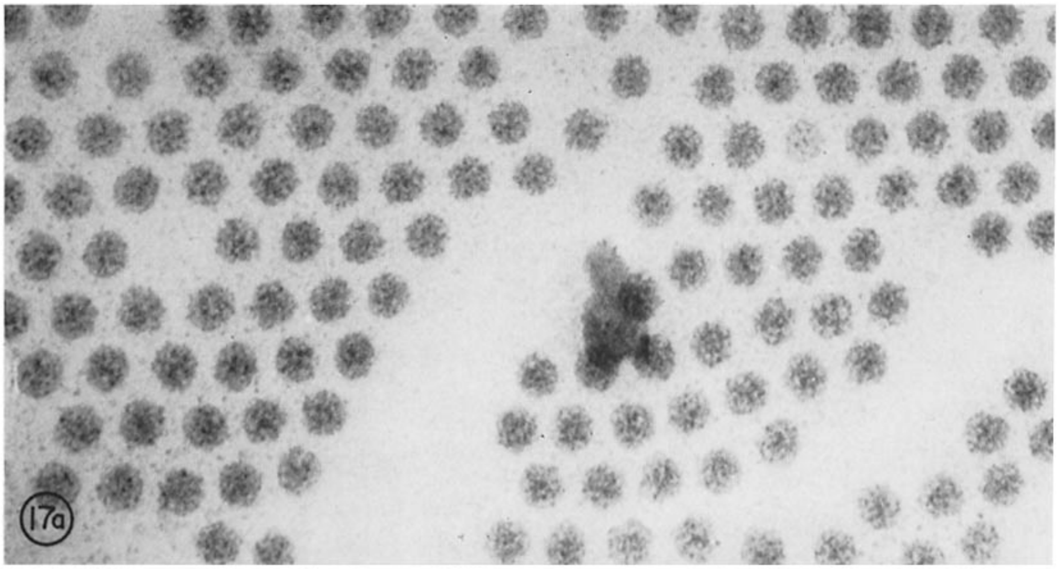
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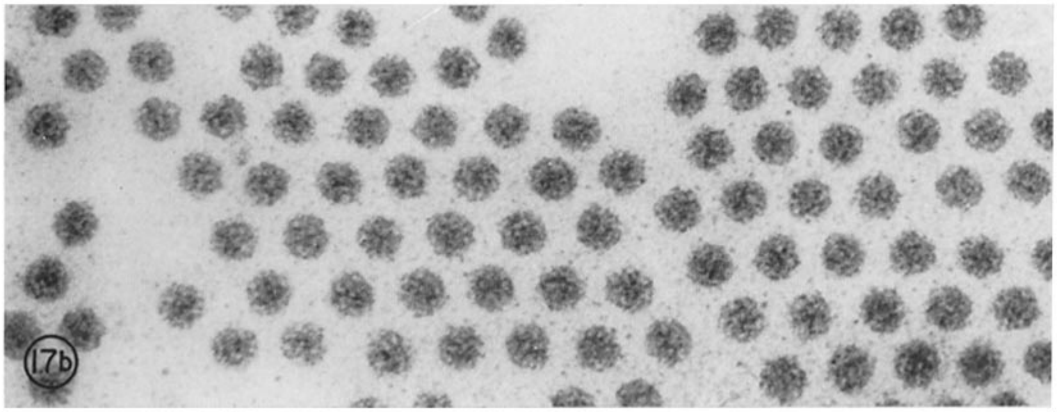
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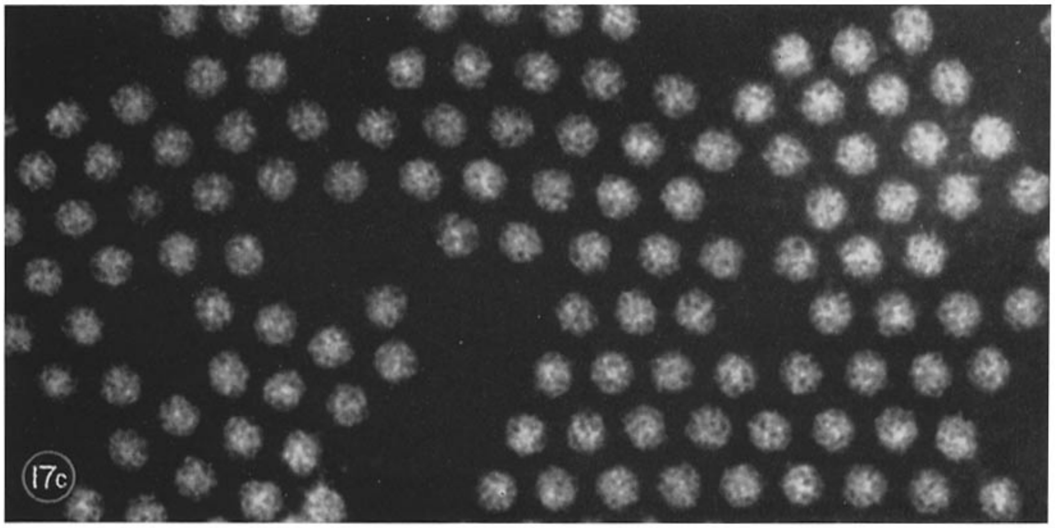
17c

as was also found, with buffered osmium fixative) is not due to any protective action of the pretreatment, for initial exposure to lead hydroxide still permits uranyl acetate staining to take place normally and a second treatment with the lead hydroxide solution then again produces a further increase in contrast. Preparations of BSV on which the combination of uranium and lead staining has been employed are illustrated in Fig. 17.

CONCLUSIONS

The observations described above show that uranyl acetate or an ion derived from it can combine with nucleic acid in amounts sufficient to increase the dry weight of the nucleic acid (DNA) by a factor of almost 2. They also show that a considerable number of instances exist where nucleic acid–containing structures in fixed cells, and in isolated, unfixed viruses, appear strongly and preferentially stained in the electron microscope after treatment with uranyl acetate solutions. They further show that many proteins, including histone, take up considerably smaller quantities of uranium than do nucleic acids, and stain only weakly or not at all. Finally, the Feulgen staining experiment indicates that at least a substantial part of the nucleic acid originally present in several tissues survives the preparative procedures and is available in the sections. It therefore appears extremely likely that uptake of uranium by the nucleic acid is responsible for at least a considerable part of the observed staining.[1]

The experiments also show that some differentiation between DNA and RNA may be possible by the comparison of "uranium-lead" and "lead only" staining in lightly fixed tissue. The exact conditions under which this differentiation is obtained optimally may very well vary from one tissue to another.

To what extent the original structure and distribution of this nucleic acid is maintained during the preparative procedures is less certain. The preservation of orientation and some structure in the purified nucleohistone is encouraging, but it does not necessarily follow that other distributions of nucleic acid, perhaps in a less condensed form, would be equally well preserved. Thus the aggregates seen in thymus nuclei could quite conceivably be artefacts. The experiments described here should be regarded as only preliminary steps towards fixation and staining of nucleic acids in whole tissues. They answer a certain number of questions, but leave very much still to be established by further experience.

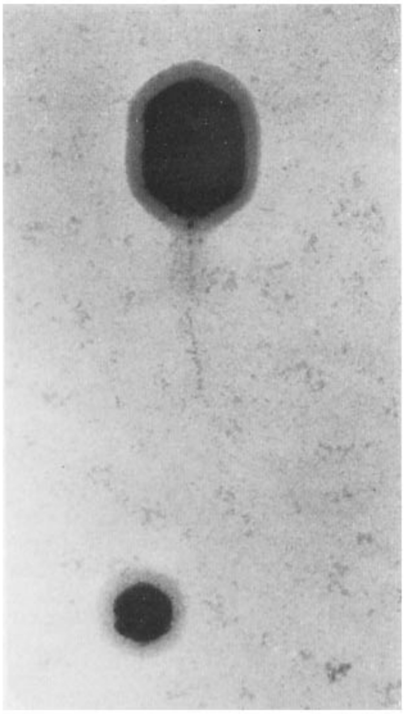

Figure 18

Phage particles (T2 and T7) stained with uranyl acetate followed by lead hydroxide. Very intense staining of these DNA-containing particles takes place, so that they become almost totally opaque to the electron beam. × 200,000.

Figure 17

BSV, stained with aqueous uranyl acetate for 6 hours, followed by lead hydroxide for $1\frac{1}{2}$ hours. The staining is somewhat stronger than that obtained by uranyl acetate alone, and the cores of the particles still show a great deal of internal detail, perhaps particularly well seen in the reversed print (*c*). × 300,000.

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[1] *Note added in Proof:* See also the recent paper on interaction of DNA with uranyl salts by Zobel, C. Richard, and Beer, Michael, *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, 335.