CHROMOSIN, A DESOXYRIBOSE NUCLEOPROTEIN COMPLEX OF THE CELL NUCLEUS

By A. E. MIRSKY and A. W. POLLISTER

(From the Laboratories of The Rockefeller Institute for Medical Research and the Department of Zoology, Columbia University, New York)

PLATE 3

(Received for publication, June 20, 1946)

Nucleoproteins can be extracted and isolated from the nuclei of animal and plant tissues by the same procedure, somewhat modified, that is effective in preparing the nucleoprotamines of sperm (1, 2). In this paper the preparation of nuclear nucleoproteins from animal and plant tissues and from bacterial cells is described and some results of an investigation of these substances are given. Nucleoproteins are also present in the cytoplasm of animal and plant tissues. It is an important step in the isolation of nucleoproteins to separate those occurring in the nucleus from those of the cytoplasm. This can be accomplished in two ways: by chemical procedures of fractionation and by methods in which well formed nuclei or chromosomes are isolated from the cytoplasm before extraction is begun. These two methods of preparation have in practice much in common. Although many bacteria may not have distinctly formed nuclei, they do have both the nucleoproteins found in the nuclei and those found in the cytoplasm of cells in which the distinction between nucleus and cytoplasm can be made morphologically. In this paper the preparation of what might be called the nuclear nucleoproteins of bacteria is described and it will be seen that this preparation was made possible by the experience gained by working with animal and plant cells. Although this paper is concerned primarily with nuclear nucleoproteins, the first steps in the isolation of cytoplasmic nucleoproteins are outlined.

Animal and plant tissues in general have not appeared to be favorable material for investigation of nuclear constituents because in so many tissues nuclei are embedded in relatively large masses of cytoplasm. For this reason Miescher (8), Kossel (3), and other leading investigators preferred to work on sperm, cells in which there is little cytoplasm. The thymus gland is an animal tissue in which nuclei form an unusually large fraction of the total volume, and on the nuclear constituents of the thymus there are the important investigations of Lilienfeld (27), Huiskamp (7), Bang (7*a*), Hammarsten (22), and others. These investigators did not use strong acid and alkali, the reagents by means of which the somewhat disintegrated nuclear constituents of sperm had been isolated; instead, in extractions from the thymus nothing more drastic than distilled water was used. The consequences of this mild treatment were that nucleic

acid and basic protein were extracted from thymus nuclei, not separately but as a nucleohistone complex, and the nucleic acid moiety of the complex was preserved in its highly polymerized state. Extraction of nuclear constituents with water has not proven to be a generally applicable procedure; it is ineffective when applied to sperm and to animal and plant tissues in general. Neither nuclear nucleoproteins nor highly polymerized nucleic acid has until recently been prepared from sperm or from animal and plant tissues in general, although depolymerized nucleic acid has of course been prepared from these sources. Histones have been extracted from certain sperm, from the thymus gland, and from avian erythrocytes (where, indeed, they were discovered by Kossel), but no success has attended the search for histones in most other animal cells; in the liver, for example, no histone could be found. Perhaps it was because of the failure to isolate histones from the nuclei of most cells that Kossel, the foremost investigator of these proteins, finally came to the conclusion that "they do not, however, occur in all nuclei, but only in the nuclei of certain kinds of tissues." (3)[1]

By the methods to be described in this paper it is possible to isolate nucleoproteins from the nuclei of many different animal tissues and also from the nuclei of plant tissues. Although the nucleoproteins are derived from tissue in which nuclei form only a small part of the total volume, it can be demonstrated that they are indeed derived from the nuclei. In each nucleoprotein the nucleic acid moiety is highly polymerized, indicating that in every instance the nucleic acid as it occurs in the cell nucleus is a high polymer. These nuclear nucleoproteins contain histones. The range of distribution of histone-containing nucleoproteins is so wide, that Kossel's view concerning the restricted occurrence of histones can now be abandoned; it now appears probable that, either histones or protamines, are constituents of most, if not all, nuclei. Histones (or protamines) and desoxyribose nucleic acid are, in our present state of knowledge, two of the distinctive constituents of chromatin.

Although the nuclear nucleoproteins we have prepared contain histones, we do not refer to them as *nucleohistones* because in addition to nucleic acid and histone these substances contain other proteins that can be distinguished sharply from histones. At present there is no reason to consider these other proteins to be impurities derived from the cytoplasm. It is generally supposed

[1] More recently it has been claimed by Caspersson (4) that histones can be detected even within the nucleus by means of their characteristic absorption in the ultraviolet, and on this basis it is supposed that "histone-type" proteins have been observed in the nuclei of many cells. The significance of these observations is at present doubtful and whatever their meaning may eventually prove to be, it seems unlikely that they indicate the presence of histone, for the ultraviolet absorption spectra of numerous purified histones that we have prepared do not resemble Caspersson's "histone-type" absorption spectrum.

that the so called nucleohistone of the thymus consists solely of nucleic acid and histone. This has never been demonstrated, and indeed many years ago Steudel (5) adduced some evidence to the contrary. We have now succeeded in isolating the non-histone protein component of the nuclear nucleoproteins and to continue to refer to them as nucleohistones would be misleading. Certainly what is most significant about the fibrous nucleoproteins derived from the nuclei of animal and plant cells is that these substances are constituents of chromatin and (as will be shown in another paper), at least in some instances constitute as much as 90 per cent of this important nuclear material. *Chromosin*[2] would, accordingly, appear to be a suitable name for the nucleoprotein complex prepared from chromatin.

The nucleoproteins we have prepared are strikingly fibrous, and they are extracted with 1 M NaCl. Extraction of exceedingly fibrous materials from tissues by means of concentrated neutral salt solutions was first accomplished many years ago and since then has been repeated again and again. The first mention of this phenomenon is found in the 2nd edition, published in 1865, of Hoppe-Seyler's "Handbuch der physiologisch—und pathologisch—chemischen Analyse." (There is no reference to the phenomenon in the first edition, published in 1858.) In this book (p. 362) a description is given of the effect of adding (to pus cells, separated by filtration from pus) a 10 per cent sodium chloride solution. A thick slimy mass was obtained which, on filtration, yielded a viscous opalescent fluid. When this fluid was poured into distilled water a fibrous precipitate formed. Hoppe-Seyler noted that the fibrous precipitate had some of the properties of myosin. From 1865 to 1941 fibrous materials similar to that obtained from pus cells were prepared from many different tissues and on each occasion they were compared with myosin and fibrin, the two familiar fibrous proteins. They were not compared with nucleohistone, and this may seem surprising since thymus nucleohistone was first prepared in 1892 and since we now know that the fibrous materials extracted from so many tissues by concentrated salt solution are in fact histone-containing nucleoproteins. The failure to compare the fibrous materials with thymus nucleohistone is to be attributed to the fact that the method (7) then used for extraction of nucleohistone from the thymus gland modified the nucleohistone so that its markedly fibrous character was lost. And yet it is curious to note how closely the fibrous material of pus cells extracted with 10 per cent sodium chloride was associated with the discovery of nucleic acid. The first half of Miescher's classical paper of 1871 (8) in which the discovery of "nuclein" was described was concerned with a study of the fibrous material extracted from pus cells with 10 per cent sodium chloride. Miescher, working in Hoppe-Seyler's laboratory, compared this material with myosin and fibrin, though he

[2] This should not be confused with the so called chromosomin of the Stedmans (6).

was by no means satisfied that this was a correct analogy. He soon turned away from study of this material to an entirely different method of investigating the composition of pus cells, and so to the discovery of nucleic acid.

The latest investigations on the fibrous material extracted from tissues by concentrated salt solutions are those of Bensley (9) and of Szent-Györgyi and his colleagues (10). Again this analogy with myosin is considered to be highly significant by these investigators. Bensley and his colleagues consider this material to form the structure of cytoplasm and, accordingly refer to it as plasmosin. Szent-Györgyi and his collaborators prepared this fibrous material from kidney and liver and refer to it as renosin and hepatosin to indicate that in the kidney and liver this substance is somewhat analogous to myosin of muscle. With the knowledge that the fibrous material extracted with 1 M NaCl is a constituent of chromatin and that its fibrous character is due to the presence of polymerized desoxyribose nucleic acid, these analogies lose their value.

Preparation

First Method.—In preparing chromosin from a tissue such as the liver the first step, after mincing the tissue, is a thorough extraction with physiological saline. The bulk of the material extracted is cytoplasmic, so that a considerable purification of nuclear constituents is accomplished while they still remain in the cell. The minced tissue is washed with physiological saline as long as material precipitable with trichloracetic acid continues to be removed. In this way 67 per cent of the substance of the liver can be washed away, and yet cytological examination of the residual material shows that the main outlines of cell structure have not been destroyed. By washing with saline at pH 9.2 as much as 82.5 per cent of the liver substance can be extracted and, in this case too, cytological examination shows an intact cell framework. Washing at pH 10.2 breaks up the cell structure so that only a mass of debris can be seen. Since thorough washing with neutral saline or saline at pH 9.2 clears out the cytoplasm to a considerable extent without any apparent effect on the appearance of the nuclei, the result is that a tissue like the liver with a low nuclear volume becomes a tissue with a nuclear volume approaching that of the thymus. The nucleus contains about 10 per cent of the mass of a liver cell. After washing with neutral saline it accounts for about 30 per cent of the cell mass and after washing at pH 9.2 the nuclear mass becomes more than 50 per cent of the cell mass. The preliminary extraction of a tissue with saline is ordinarily done with neutral saline.

Among the cytoplasmic constituents removed from a tissue by washing with saline are cytoplasmic nucleoproteins. Since the nuclear nucleoproteins do not dissolve in physiological saline, it is possible in this way to obtain an extract of cytoplasmic nucleoproteins without any considerable admixture of nuclear constituents. And when the nuclear nucleoproteins are extracted subsequently, the extract will not at the same time contain the bulk of the cytoplasmic nucleoproteins. In the cell the two types of nucleoprotein are in large

measure located separately, and in preparing them it is simpler and more effective to extract them separately than to mix them and later attempt to fractionate.

After washing with saline the tissue is extracted with 1 M NaCl. Immediately on adding the concentrated salt solution the mixture becomes exceedingly viscous, a sign that chromosin is passing into solution. After vigorous and prolonged stirring the mixture is centrifuged. Because of the high viscosity of chromosin solutions, clarification occurs only if centrifugation is at fairly high speed (approximately 10,000 R.P.M.). The extracted chromosin is precipitated by pouring the solution into 6 volumes of water, which reduces the NaCl concentration to 0.14 M. Chromosins are insoluble in 0.14 M NaCl and soluble in 1 M NaCl, so that by alternately adding concentrated salt solutions and diluting with water, these substances can be repeatedly dissolved and precipitated. Precipitation of a chromosin occurs in long fibrous strands which can be wound around a rod and (if the rod has a crook at its end) readily lifted out of one vessel and placed into another. In this way the fibrous precipitate of chromosin is easily separated from impurities, which, though insoluble in 0.14 M NaCl, are granular rather than fibrous in character.

Chromosins have been prepared from the following animal tissues by this method: mammalian liver, kidney, thymus, spleen, pancreas, brain; frog testes; avian erythrocytes; fish liver and erythrocytes. The preparations from bird and fish erythrocytes will be described in detail in another paper. Unheated wheat germ is the plant material from which a chromosin has been isolated. The preparative procedure differs in several respects from that used for animal tissues: the wheat germ is thoroughly extracted with petrol ether and then dried at room temperature before being washed with 0.14 M NaCl; and the solution of chromosin in 1 M NaCl is poured into 10 volumes of water to precipitate chromosin, instead of into 6 volumes. 50 gm. of wheat germ are used for one preparation.

The bacteria from which chromosin was prepared were Type III pneumococci. The suspension of heat-killed bacteria used in the first preparation was given to us by Dr. Avery. For subsequent preparations the pneumococci were grown by a slightly different procedure from that followed in Dr. Avery's laboratory. Although no nucleus is detectable in a pneumococcus cell, the cells are thoroughly washed with physiological saline in the same way as that in which liver and other tissues are to remove cytoplasmic constituents. In this way the bulk of the ribose nucleoproteins (in other cells located mainly in the cytoplasm) is removed, and when the desoxyribose nucleoproteins (in other cells located in the nucleus) are later extracted and precipitated they are found to be almost entirely free of ribose nucleoprotein. Because of the effectiveness of a preliminary removal of "cytoplasmic nucleoproteins" in the isolation of pneumococcal desoxyribose nucleoproteins by us, this procedure was subsequently used in the isolation of the transforming principle by Avery,

MacLeod, and McCarty (11). In the preparation of chromosin from pneumococci the 1 M NaCl extract is not added to 6 volumes of water because so little material is present in the extract. Instead the extract is dialyzed against 0.14 M NaCl and in the course of dialysis threads of chromosin are seen to appear within the cellophane dialysis tube.

For the extraction of chromosin from pneumococci the dry weight (not including capsular material) of the bacteria should amount to no less than 3 gm. Because of the small quantity of chromosin extracted the volume of the bacterial suspension in 1 M NaCl should not exceed 100 cc. After the preparation of chromosin in the cellophane tube the chromosin is redissolved in approximately 5 cc. of 1 M NaCl and from this point on the material is treated just as is chromosin isolated from animal tissues.

Attempts to prepare chromosin from the colon bacillus and from hemolytic streptococci have not been successful.

The preparation of chromosin from calf liver will be described in detail. All operations are, unless otherwise stated, carried out in a cold room maintained at 0–1°C. Within an hour after being removed from the calf 300 gm. of liver are cut into fairly small pieces and suspended in 1 liter of 0.14 M NaCl. The liver suspension is minced in a high speed electric mixer (Waring blendor) for 1 minute. A drop of octyl alcohol is added to cut down the foam and the suspension is then centrifuged for 10 minutes at 8000 R.P.M. The turbid supernatant is discarded and the residue is again suspended in 1 liter of saline. The suspension is mixed by being placed in the Waring mixer for a few moments. After centrifuging at 8000 R.P.M. the supernatant is found to be much less turbid than it was the first time. The residue is washed twice more. The residue from the final washing is suspended in 0.14 M NaCl, final volume 750 ml. To this suspension are added 750 ml. of 2 M NaCl. This suspension is placed in the Waring mixer for 2 to 3 minutes. The mixture, now quite viscous, is stirred rapidly for 16 hours. On centrifuging for 60 minutes at 8000 to 10,000 R.P.M. a fairly clear, viscous, supernatant is obtained. This solution contains chromosin. Chromosin is precipitated from solution by pouring into 6 volumes of water. A ropy precipitate is obtained. This quickly settles and the opalescent supernatant is decanted. To the precipitate is added an equal volume of 2 M NaCl and 1 M NaCl is added to bring the total volume to about 450 ml. Vigorous stirring dissolves the chromosin. Undissolved material is removed by centrifuging at 8000 to 10,000 R.P.M. for 60 minutes. The supernatant is poured into 6 volumes of water and this time the fibrous material is wound around the stirring rod, which has a crook at its end, and so transferred to another vessel. The chromosin is dissolved and precipitated 3 more times. Dissolved in 1 M NaCl, the chromosin keeps well in the cold without a preservative.

The fibrous nucleoproteins described in this paper have been referred to as chromosins because it is believed they are of nuclear origin, being derived, indeed, from the chromatin of the cell nucleus. The evidence for the nuclear origin of these nucleoproteins will now be given:

1. Chromosins closely resemble both in general properties and in composition the fibrous nucleoproteins prepared from sperm, and there can be no doubt that the latter are derived from the sperm nucleus.

2. The quantity of chromosin extracted depends upon the relative sizes of nucleus and cytoplasm in each tissue. From a given mass of tissue far more chromosin is extracted from the thymus, for example, than from liver, and whereas the nucleus forms about one-tenth the volume of a liver cell, it forms at least several times that part of a thymus lymphocyte.

3. Chromosins, it will be shown in this paper, can be extracted from the isolated nuclei of tissue cells.

4. Chromosins can be prepared from isolated chromosomes and, at least in one instance, a chromosin forms as much as 90 per cent of the mass of the chromosomes (12).

5. Chromosins contain considerable quantities of desoxyribose nucleic acid and for this reason give intensely positive Feulgen reactions. Many careful cytological investigations with the Feulgen technique have been made, and it is generally agreed that the only part of a cell to react positively is the nucleus. Precisely how much desoxyribose must be present in a microscopic preparation before it reacts positively to the Feulgen reaction is not known, so that all that can be said is that little, if any, desoxyribose nucleic acid is present in cytoplasm. Feulgen staining shows, therefore, that most, if not all, of the chromosin extracted from a cell is derived from the nucleus.

6. A cytological study of the effect on frozen sections of liver tissues of the procedure for chromosin extraction demonstrates clearly that the latter comes from the cell nuclei. The result of treatment of the frozen sections with physiological saline, over a pH range from 7.0 to 9.2, is to remove a major part of the stainable substance of the cytoplasm leaving the nucleus unchanged (Fig. 4); and thus it appears that the considerable amount of material which the preliminary wash extracts from homogenized liver comes mainly from the cytoplasm. By contrast the second step which involves placing the washed section in 1.0 M sodium chloride, causes the nuclei to swell and to become non-staining vesicles (Fig. 5). That the chromatin in such swollen nuclei has actually become a solution of chromosin in 1.0 M sodium chloride can be clearly demonstrated by returning the section to weak saline (as in step 3 of the extraction procedure) which precipitates the chromosin fibers. These intranuclear fibers are basophilic and Feulgen-positive, and are thus identical in staining properties with the original nuclear chromatin and also with the chromosin fibers which can be wound around a glass rod in the course of the mass extraction procedure. One seems justified in considering that these experiments on unfixed slices of liver demonstrate on a microscopic scale within each nucleus the very sequence of events that is observed on a gross scale during the preparation of chromosin from a large mass of homogenized liver tissue. This cytological evidence

completely agrees with the chemical and other proof that the cell nucleus is the major, if not indeed the only, source of chromosin. The most extensive series of experiments of the sort described above were made on liver sections, but the significant results have also been obtained with kidney, pancreas, and testis. Laskowski and Ryerson have furnished confirmation from a study of the effect of salt solutions upon avian erythrocytes (13).

Second Method of Preparing Chromosin.—In this method the preliminary removal of cytoplasmic constituents is far more thorough than the first method; in this method the whole cytoplasm is removed, leaving isolated nuclei and from them chromosin is extracted with 1 M NaCl. The chromosin prepared by this method is obviously less likely to contain cytoplasmic impurities than is the chromosin prepared by the first method. The disadvantages of the procedure involving isolation of nuclei are: (1) it has not been possible to isolate the nuclei of some tissues, not those of the brain, for example; (2) the quantity of chromosin extracted from the isolated nuclei of some tissues is very low; and (3) isolation of nuclei involves the use of citric acid, and in some cases the use of even a weak acid is undesirable. When chromosin is extracted from pneumococci, for example, use of acid should be avoided, if the material is being prepared for an investigation of the transformation of pneumococcal types.

Isolation of nuclei is accomplished by the citric acid method of Crossmon (14) and Stoneburg (15). To obtain suspensions of clean nuclei we have found it necessary to modify the original procedure. Citric acid disintegrates the cytoplasm and at the same time tends to fix the nucleus. Nuclei can be separated from the mass of disintegrated cytoplasm by low speed centrifuging because they are much heavier than most of the cytoplasmic débris; and nuclei can be separated from connective tissue fibers and other large particles by straining, for nuclei are small enough to pass through even the finest muslin. By a combination, therefore, of centrifuging and straining it is possible to prepare a suspension of nuclei that appear clean when examined microscopically. A microscopic examination of the nuclei should be made after citric acid has been washed away and the nuclei are suspended in neutral saline, for connective tissue fibers swollen in citric acid may become visible only after the suspension has been neutralized. Clean nuclei have been prepared from liver, thymus, pancreas, and kidney (Figs. 1 and 2). Stoneburg isolated nuclei from muscle and tumor tissues but was unable to do so from the thymus and liver. Subsequently Marshak (16) and Dounce (17) isolated nuclei from liver by the citric acid method. We were unsuccessful in our attempts to isolate nuclei from the brain.

While suspended in citric acid, the nuclei keep in the cold for at least 4 or 5 days. When neutralized in saline they do not keep well because of a rapid

autolysis that occurs even in the cold,[3] and as a result of which the nuclei soon clump together in large gelatinous masses.

Chromosins have been prepared from liver and thymus nuclei. The nuclei are neutralized with phosphate buffer or with bicarbonate and then extracted with 1 M NaCl. Once the chromosin is extracted from the nuclei, it is isolated in just the same manner as when the extraction is from tissue washed from saline.

The preparation of nuclei from the thymus will be described in detail. All operations are carried out in a cold room kept close to 1°C. 100 gm. of fresh calf thymus are minced with scissors, suspended in 500 ml. of 1 per cent citric acid, and placed in a Waring mixer for 6 minutes. A drop of octyl alcohol is added to cut down the foam and the mixture is centrifuged for 10 minutes at 3500 R.P.M. The supernatant is discarded. The precipitate, resuspended in 750 ml. of 0.2 per cent citric acid, is stirred at low speed (obtained by means of a rheostat) in the mixer for a short time to break up the larger particles. The suspension is strained through a double layer of finely woven towel and then centrifuged at 3500 R.P.M. for 5 minutes. 750 ml. of 0.2 per cent citric acid are again added to the precipitate which is again broken up by low speed stirring in the mixer. This time the suspension is strained through a double layer of the finest muslin and then centrifuged at 1200 R.P.M. for 5 minutes. No more straining is required, but the nuclei are washed with 0.2 per cent citric acid by alternately mixing at low speed and centrifuging at low speed until the supernatant is clear. No more than three washings after the last straining should be necessary. If the nuclei are to be kept, this is best done while they are suspended in 0.2 per cent citric acid. The first step in the preparation of chromosin is to wash the nuclei several times in a 0.14 M NaCl – 0.01 M pH 6.8 phosphate buffer. While the nuclei are suspended in this medium an equal volume of 2 M NaCl is added. The material is stirred at fairly high speed in the Waring mixer for about 3 or 4 minutes and then with an ordinary stirrer (using a stirring rod) overnight. In the morning the mixture is centrifuged at 8000 R.P.M. for 75 minutes. The slightly opalescent, viscous supernatant contains chromosin.

Composition.—For analysis the precipitated chromosin is washed first with 65 per cent alcohol, then with hot 95 per cent alcohol, and finally with ether. It is dried at 106°C. It is necessary to precipitate a chromosin and so remove the bulk of the sodium chloride in which it is dissolved before adding alcohol. If alcohol is added to chromosin while it is still dissolved in 1 M NaCl much (and how much depends on the pH of the medium) of the histone will be dissolved in the alcohol. Treatment with alcohol and ether removes lipids that might be adhering to the chromosin. In the present investigation such lipids have not been considered. Phosphorus is estimated by Allen's method (18) and nitrogen by the method of Koch and McMeekin (19).

[3] Results obtained in an investigation of nuclear autolysis will be published separately.

The chromosins described in this paper all have approximately the same nitrogen and phosphorus contents. There is, furthermore, no evidence that the composition varies with the method of preparation. Variations in composition are as great in different preparations from the same tissue as they are in preparations from different tissues. Phosphorus contents lie between 3.55 per cent and 4.30 per cent; nitrogen contents lie between 14.2 per cent and 16.3 per cent. All of these chromosins contain histones. Protamine-containing chromosins contain both more phosphorus and nitrogen. In the nucleoprotamine prepared from trout sperm, for example, there are 6.10 per cent phosphorus and 18.3 per cent nitrogen.

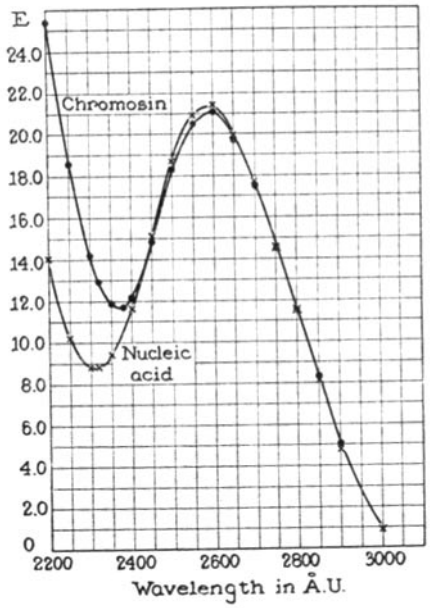
There are three components in a chromosin: desoxyribose nucleic acid, histone (or protamine), and non-histone protein.[4] The recognition and isolation of each will be described.

Nucleic Acid.—Nucleic acid can be isolated from a chromosin but even before it is isolated it can be detected and characterized quantitatively. The absorption spectra in the ultraviolet of the chromosins we have prepared show that these substances contain nucleic acid, and furthermore they show that all of the phosphorus of a chromosin is in the form of nucleic acid. In Text-fig. 1 are shown the absorption curves of a chromosin and of nucleic acid. It can be seen that chromosin has an absorption spectrum quite similar to that characteristic of nucleic acid. It can also be seen that when the extent of absorption is expressed in relation to phosphorus content that the absorption coefficient at maximum absorption (2600 A. u.) is the same for chromosin and nucleic acid. At this wave-length presence of protein in chromosin has little effect on absorption. At longer wave-lengths also the absorption due to nucleic acid is so much more intense than that due to protein that the chromosin absorption curve is very close indeed to that of nucleic acid. The effect of the protein present in chromosin on the over-all absorption can be clearly perceived at wave-lengths shorter than 2450 A. u. because in this region absorption by protein is by no means negligible compared with that by nucleic acid. In the region below 2450 A. u., chromosin, due to its protein content has more intense absorption than has an equivalent quantity of nucleic acid; and chromosin has a point of minimum absorption at 2370 A. u. instead of at 2300 A. u. for nucleic acid. The absorption spectrum of chromosin shows that it contains nucleic acid, but it does not tell us which nucleic acid it contains, for absorption is due to the purine and pyrimidine bases of nucleic acid and these are so nearly alike in ribose and desoxyribose nucleic acids that the two known types of nucleic acid cannot be distinguished by their ultraviolet absorption spectra.

By means of the diphenylamine reagent of Dische (20) it can be shown that all of the nucleic acid in a chromosin is of the desoxyribose type. Large quan-

[4] In the chromosin prepared from trout sperm only desoxyribose nucleic acid and protamine were found (2).

tities of protein are known to interfere with the diphenylamine reaction, but the quantity present in a chromosin apparently introduces no difficulty. If the intensities of blue color given with diphenylamine by a sample of desoxyribose nucleic acid and by chromosins of the same phosphorus content are measured, it is found that they are the same. Since ribose nucleic acid does not react with the diphenylamine reagent, this is evidence that in a chromosin the ratio of



TEXT-FIG. 1. Ultraviolet absorption curves of thymus nucleic acid and thymus chromosin dissolved in M NaCl. The extinction coefficients of both solutions are for concentrations of nucleic acid of 1 mg. per ml. The effect of protein on the absorption of chromosin is apparent only at wave-lengths shorter than 2450 A.u.

desoxyribose nucleic acid to phosphorus is the same as it is in pure desoxyribose nucleic acid, and that, at the most, 3 or 4 per cent of the nucleic acid in a chromosin can be of the ribose type.

The value of the phosphorus-desoxyribose ratio as a criterion of purity is strikingly shown in the case of chromosin prepared from the pancreas. In this tissue it has been estimated that there are eight times as much ribose as desoxyribose nucleic acid. And yet the ratio of phosphorus to desoxyribose found for pancreas chromosin is the theoretical ratio for a desoxyribose nucleic acid, indicating that little, if any, ribose nucleic acid is present in this chromosin.

The diphenylamine reaction can give us more than a determination of the desoxyribose content of the nucleic acid in a chromosin. In another paper

it will be shown that this reaction also gives us the ratio of purine to pyrimidine nucleosides in either isolated desoxyribose nucleic acid or in chromosin. These nucleosides are present in a 1:1 ratio in every chromosin that we have prepared.

Nucleic acid can be isolated from a chromosin by shaking with a chloroform-octyl alcohol mixture (21). A solution of chromosin in 1 M NaCl is shaken with half its volume of a 4:1 chloroform-octyl alcohol mixture. After shaking vigorously for about 5 hours the mixture is centrifuged and the upper of the 3 layers is removed and again shaken with chloroform-octyl alcohol. The upper layer contains nucleic acid to which some protein is still attached; the lower layer consists of chloroform-octyl alcohol; and the middle layer contains a precipitate of protein that has been separated from nucleic acid. The phosphorus content, 0.05 per cent of the material in the middle layer, shows how free of nucleic acid it is. After the shaking process has been repeated 6 or 7 times the middle layer contains only a slight amount of protein. The nucleic acid still contains a little protein, but this can be removed by adding the solution to 5 volumes of alcohol. A fibrous precipitate is obtained. This is washed with alcohol, ether, and dried. It contains 8.9 per cent phosphorus and 15.4 per cent nitrogen and has a N:P ratio of 1.73. The theoretical composition of the sodium salt of desoxyribose nucleic acid is: 9.28 per cent P; 15.58 per cent N; and the N:P ratio is 1.68. The dried fibers of nucleic acid readily dissolve in water to form a clear viscous solution which exhibits birefringence when stirred. Its properties resemble those of the nucleic acid prepared from the thymus gland by the Bang-Hammarsten procedure (22). It is therefore possible by first isolating chromosins to prepare pure, highly polymerized desoxyribose nucleic acid from many animal and plant tissues. Hitherto such nucleic acid has been prepared from the thymus gland only.

Histone.—Histone is split from a chromosin with hydrochloric acid. The fibrous precipitate is placed in a mortar and as much water as possible is pressed out of it. To the fibrous mass is added 0.2 N HCl and the mixture is ground from time to time for an hour. The suspension is centrifuged, giving a clear solution of histone in hydrochloric acid. The HCl is removed by dialysis. That the protein in solution is indeed a histone is shown by adding dilute ammonium hydroxide to the solution; a precipitate is obtained which does not redissolve in an excess of NH_4OH. It was this property of a histone by which it was originally characterized by Kossel (3). The pH at which precipitation occurs has been measured for both calf liver and wheat germ histones. Measurements of pH were made with the glass electrode and in the region above pH 10 determinations are subject to error. The solution of liver histone becomes opalescent at pH 10.72 and a precipitate occurs at pH 10.83. The solution of wheat germ histone (which was not dialyzed to remove hydrochloric acid) becomes turbid at pH 10.09 and a definite precipitate forms at pH 10.41.

The isoelectric points of liver and wheat germ histones are probably in the neighborhood of 10.8 and 10.4 respectively.

Histone can be prepared directly from isolated nuclei, without first isolating chromosin. A suspension of nuclei in citric acid is concentrated by sedimentation in the centrifuge and then treated with 0.2 N HCl. Histone, and no other protein, is in this way extracted from the nuclei.

If a histone preparation is to be kept for some time, it is not dialyzed free of hydrochloric acid. Instead sodium hydroxide is added to the histone solution until massive precipitation of histone occurs. Precipitation begins soon after neutralizing and alkali is added until a pH of approximately 10 is reached. Two volumes of 95 per cent alcohol are then added, and the suspension is allowed to settle overnight. The precipitated histone is gathered by centrifugation. After thorough washing with alcohol and ether the protein is dried at room temperature. The dried histone dissolves completely and at once in dilute HCl.

We have found, in addition to their basic properties, several other properties of histones which distinguish them from most other proteins: (1) The histones that we have prepared contain no more than traces of tryptophane. The low tryptophane content of histones serves as a useful guide in isolating chromosins. As a chromosin is purified it is found that the histone fraction prepared from it gives a progressively less intense test for tryptophane. (2) Histones are *not* precipitated by divalent mercury in presence of a strong acid. When a solution of almost any protein, a serum protein for example, is added to an equal volume of 0.34 M $HgSO_4$ in 1.88 M H_2SO_4 and the mixture is warmed at 60° the protein precipitates, but if the protein in solution is a histone no precipitate appears. The non-precipitability of histone by the $HgSO_4$—H_2SO_4 reagent makes it possible to tell when other proteins are mixed with a histone. A $HgSO_4$—H_2SO_4 solution is a useful medium for detaching histone and no other protein from a chromosin for quantitative estimation. These and other properties of histones will be described in detail in another paper.

The Non-Histone Protein of Chromosins.—The properties of histones that have just been mentioned make it possible to show that although histones may constitute the bulk of the protein in a chromosin, not inconsiderable quantities of other proteins are also present. When histone is split off a chromosin by means of dilute HCl, much protein still remains attached to the nucleic acid. The fibers of a chromosin prepared from thymus nuclei were extracted repeatedly with 0.2 N HCl until no more histone could be extracted, and the residue was then dehydrated with alcohol and ether and dried. The phosphorus content of the residue was only 6.15 per cent (to be compared with a content of 9.89 per cent for protein-free nucleic acid). That the residue contained protein was shown by the fact that it gave an intense Millon reaction and on analysis

proved to contain 1.02 per cent tyrosin. In his work on thymus nucleohistone Steudel observed something like this many years ago and was unable to decide whether the protein that remains attached to nucleic acid is a non-histone protein, or simply some histone that has failed to be detached from nucleic acid (5). Although there was some evidence that the residual protein differs from histone, what puzzled Steudel was that if he added pure histone to nucleic acid much of it remained attached to the nucleic acid when he treated the synthetic nucleo-histone with dilute HCl.

To discover whether a chromosin contains any protein in addition to histone, a procedure is needed for removing practically all the protein from the nucleic acid of a chromosin. This can be accomplished by adding sufficient alkali to a chromosin solution in 1 M NaCl to raise the pH to between 10 and 11 and then shaking this solution with a chloroform-octyl alcohol mixture. After prolonged shaking the whole protein of the chromosin is concentrated as an insoluble precipitate at the interphase between water and the chloroform-octyl alcohol mixture, leaving the nucleic acid dissolved in the aqueous phase. Analysis of this "whole" protein shows that it contains some protein in addition to histone. The "whole" protein prepared from thymus chromosin contains 15.8 per cent N, whereas thymus histone has a nitrogen content of 16.8 per cent. The "whole" protein contains, 0.20 per cent tryptophane whereas thymus histone has only 0.038 per cent. These analyses show that in the "whole" protein there is a protein that contains less nitrogen and more tryptophane than does pure histone. Presence of a histone and a non-histone fraction in the "whole" protein is also demonstrated by treating the "whole" protein with a solution of $HgSO_4$ in dilute H_2SO_4. On the preceding page it was stated that although most proteins are precipitated by this reagent, histones are soluble in it. When the precipitate of "whole "protein is mixed with $HgSO_4$—H_2SO_4 most of the protein dissolves, leaving a not inconsiderable, insoluble residue.

The protein prepared from chromosin can be separated into two fractions, one of which consists of histone and the other of non-histone protein. Fractionation is carried out by extracting the precipitate of mixed protein with 0.2 N HCl. In this way histone is removed, leaving a residue which amounts to 27 per cent of the mixed protein. The nitrogen content of the residual protein is 14.9 per cent (that of the mixed protein being 15.8 per cent), and its tryptophane content is 0.83 per cent (that of the mixed protein being 0.20 per cent). The tryptophane of this protein accounts for practically all of the tryptophane in the mixed protein. The tryptophane-containing protein does not dissolve at all in $HgSO_4$—H_2SO_4. These experiments show that the mixed protein of chromosin consists of 2 fractions: a histone, lacking tryptophane, rich in nitrogen, and soluble in $HgSO_4$—H_2SO_4; and a fraction containing tryptophane, relatively low in nitrogen, and insoluble in $HgSO_4$—H_2SO_4.

The tryptophane-containing protein (Tr.Pr.) is an actual constituent of chromatin; it is not an impurity picked up from the cytoplasm. The thymus

chromosin of which this protein forms a part was prepared from isolated nuclei and more recent experiments on a chromosin extracted from isolated chromosomes (derived from thymus lymphocytes) show that this chromosin too contains the Tr.Pr.

Occurring in the presence of histone and nucleic acid the Tr.Pr. could be attached to either one, depending on its acid-base properties. In a neutral medium a protein with an isoelectric point in the range of pH 5 or 6 can readily combine with a base, which a histone is in neutral medium, and cannot combine with nucleic acid. A protein with an isoelectric point above pH 7 on the other hand does not combine with histone in neutral medium but does combine with nucleic acid. The Tr.Pr. was tested for its capacity to combine with both histone and nucleic acid. At pH 6.8 it did not combine with any histone added to it, but did combine with 3.4 per cent of its weight of nucleic acid. It would therefore seem to be possible that in chromatin the Tr.Pr. is combined with nucleic acid.

Tryptophane-containing protein fractions have been isolated from other chromosins. Only where the chromosin has been prepared from isolated nuclei, however, is it certain that the non-histone protein is not, at least in part, of cytoplasmic origin. Chromosins have been prepared from isolated calf liver nuclei and chicken erythrocyte nuclei, and from each chromosin a Tr.Pr. was prepared by the same method as from thymus chromosin. It need hardly be said that the method of preparation probably produced drastic changes in the Tr.Pr.

For experiments on the non-histone protein of chromosins, preparations from isolated nuclei or chromosomes were used. In the experiments to be described in detail the chromosin was prepared from isolated thymus nuclei. This chromosin solution contained 4.4 mg. of nucleoprotein per ml. When dried its phosphorus content was 3.69 per cent and its nitrogen content 14.1 per cent. To the chromosin solution enough dilute NaOH solution was added so that when 0.5 ml. of chromosin was added to 3 ml. of water no precipitate was obtained. The pH was between 10 and 11. In this way to 140 ml. chromosin were added 5.6 ml. of 0.2 N NaOH and then 70 ml. of 1 M NaCl and 200 ml. of a 4:1 chloroform-octyl alcohol mixture. This was shaken in the cold for 48 hours. After prolonged centrifuging at 3500 R.P.M. the top layer was carefully removed. To it was added enough 0.2 N NaOH to restore the pH to 10 to 11, and this solution was again shaken with chloroform-octyl alcohol. After centrifuging only a scanty precipitate was obtained between the 2 layers of liquid. Shaking and centrifuging were repeated again and this time only a very scanty precipitate formed. The N:P ratio of the solution was now 1.72 indicating that the nucleic acid was nearly protein-free.

The collected precipitates were thoroughly washed with 0.14 M NaCl containing enough NaOH to bring the pH to 10 to 11 in order to remove as much nucleic acid as possible from the precipitated protein. At this time the protein precipitate was swollen and bulky because of the chloroform in it. Washing with alcohol removed the chloroform. The nitrogen content of a dried sample of this protein ("whole

protein") was 15.7 per cent, and the tryptophane content, determined by the method of Shaw and McFarlane (23), was 0.20 per cent.

Presence of 2 protein fractions in "whole protein" was detected by adding to 1 ml. of the suspension 1 ml. of 0.34 M $HgSO_4$ in 1.88 M H_2SO_4, heating at 60° for 15 minutes, and then adding 0.1 ml. of 1 per cent $NaNO_2$ (Millon reaction). A clear red supernatant and a red precipitate were obtained, the former being due to histone. The whole protein was thoroughly extracted in the cold with 0.2 N HCl. After extraction of the suspension, 27.5 per cent of the protein remained. This protein was histone-free, for after the Millon reaction red pigment was present in the precipitate only. This protein contained 14.9 per cent N and 0.90 per cent tryptophane. It will be referred to as Tr.Pr. "Whole protein" and Tr.Pr. were also prepared from chromosins derived from isolated calf liver nuclei and fowl erythrocyte nuclei. The "whole protein" of liver chromosin had 0.40 per cent tryptophane while the Tr.Pr. had 0.94 per cent. Liver histone contains 0.09 per cent tryptophane. "Whole protein" of fowl erythrocyte chromosin had 0.08 per cent tryptophane and the corresponding histone 0.02 per cent.[5]

Thymus Tr.Pr. was washed with 0.14 M NaCl to remove acid and then suspended in saline. It was quite gelatinous. It was now tested for its ability to combine at pH 6.8 with histone and nucleic acid. The Tr.Pr. of suspension contained 3.0 mg. of protein, by dry weight, per ml. To 1 ml. of this suspension were added 1 ml. of 0.34 M $KHPO_4$ pH 6.8 and 1 ml. of a histone solution, containing 4.1 mg. of thymus histone per ml. of water. The suspension was stirred at room temperature for 30 minutes and then centrifuged. The histone concentration of the supernatant was determined by adding to 2 ml. an equal volume of $HgSO_4$—H_2SO_4, heating to 60° for 15 minutes, adding 0.2 ml. of 1 per cent $NaNO_2$, and then heating at 60° for 10 minutes. In this way a derivative of histone is made with an absorption band at 3540 A. u. Intensity of absorption at this wave-length was compared, using a Beckman spectrophotometer, with that of a histone solution treated in precisely the same way except that saline instead of Tr.Pr. in saline was mixed with it. There was no difference in the readings, indicating that no histone combined with Tr.Pr.

The same Tr.Pr. suspension was mixed with nucleic acid at various pH. The desoxyribose nucleic acid used, a preparation of the sodium salt from the thymus, was dissolved in water to give a viscous solution, containing 0.56 mg. per ml. To 1 ml. of Tr.Pr. (3.0 mg. protein) were added 2 ml. of 0.14 M NaCl, varying volumes of 0.04 N NaOH, 1 ml. of nucleic acid, and enough saline to bring the volume to 5.0 ml. When a mixture contained saline instead of nucleic acid, it was obvious on comparing them that addition of nucleic acid caused a change in state of aggregation, tending to agglutinate the suspended protein. The Tr.Pr.-nucleic acid mixtures remained at room temperature with occasional mixing for 30 minutes and were then centrifuged. The pH and nucleic concentration of each supernatant were determined, the former with the glass electrode and the latter by measurement of the extinction coefficient at 2600 A. u., using the Beckman spectrophotometer. Results are given in Table I.

[5] *Note Added to Proof.*—We have recently been checking our tryptophane determinations by other methods and find that the results reported in this paper are too low. The correct values for the tryptophane contents of Tr.Pr. and of some of the histones are higher than those given in this paper.

Pneumococcus Chromosin.—The chromosin isolated from Type III pneumococci was extracted in the same way and has the same general properties as the chromosins extracted from other cells, but because the bacterial chromosin has been available in only very small amounts, investigation of its composition has been incomplete. Some facts concerning its composition are, however, clear: the nitrogen-phosphorus ratio of 3.9 is about the same as that for many chromosins; and the ratio of desoxyribose (as given by the diphenylamine reaction) to phosphorus is that characteristic of other desoxyribose nucleic acids, indicating that the ratio of purine to pyrimidine desoxyribose nucleosides in the nucleic acid of pneumococci is the same as in the desoxyribose nucleic acids of other cells.

There is evidence that both histone and non-histone proteins are present in pneumococcus chromosin, although no protein component has been isolated.

TABLE I

pH	Quantity of nucleic acid combined	Quantity of nucleic acid in Tr.Pr.-nucleic acid combination
	mg.	*per cent*
6.2	0.124	4.15
6.5	0.103	3.42
6.8	0.101	
7.6	0.104	
9.2	0.059	1.97

For other chromosins it has been shown that a solution of $HgSO_4$ in dilute H_2SO_4 dissolves and extracts the histone, leaving a tryptophane-containing, non-histone protein attached to nucleic acid. When fibers of pneumococcus chromosin are treated with the $HgSO_4$—H_2SO_4 reagent, much of the protein is dissolved, but some remains attached to the nucleic acid fibers. It would seem likely that in pneumococcus chromosin, as in other chromosins, the soluble protein fraction consists of histone and the insoluble fraction consists of a tryptophane-containing non-histone protein.

Pneumococcus chromosin has been tested for its ability to transform the type of pneumococci. It was found that less than 0.02 gamma of chromosin added to 2.25 ml. of a culture of "rough" bacteria derived from a Type II strain sufficed to transform cells to Type III. (We are indebted to Dr. McCarty for making this test.) A preparation from Type III pneumococci of material containing desoxyribose nucleic acid that is active in transforming "rough" pneumococci to Type III has recently been described by Avery, MacLeod, and McCarty (11). It is of interest to compare this material with pneumococcus chromosin.

The method of preparation used by Avery and his colleagues and that used

in preparing a chromosin differ in certain respects, although they have much in common. In the former the material is extracted from pneumococci by means of desoxycholate, whereas M NaCl is used to extract chromosin. Desoxycholate was first used by Alloway (24) to extract the transforming principle of the pneumococcus and his reason for using desoxycholate was that it dissolves pneumococci. Later when the pneumococci were heat-killed desoxycholate no longer dissolved them, but desoxycholate continued to be used. Its real function is to dissolve the desoxynucleoprotein complex of the bacteria. This can be shown by adding a 0.5 per cent solution of neutral sodium desoxycholate to some fibers of thymus chromosin; they dissolve at once to form a clear viscous solution. Desoxycholate is a detergent, and in this case it appears to be acting as such, for a 0.5 per cent solution of another detergent, sodium dodecyl sulfate (Duponol) is also found to dissolve chromosin fibers. Desoxycholate can be used to extract the desoxynucleoprotein complex of animal tissues. When minced thymus, for example, which has previously been washed with physiological saline, is extracted with a 0.5 per cent solution of desoxycholate and then centrifuged at high speed, a clear viscous supernatant is obtained. When this is added to 9 volumes of alcohol a stringy precipitate forms, resembling that obtained under the same conditions by Alloway and later by Avery and his colleagues from pneumococci.

An advantage that desoxycholate possesses as an extracting agent when compared with M NaCl is that it extracts several times as much desoxynucleic acid from pneumococci as does M NaCl. After a mass of pneumococci has been repeatedly extracted with M NaCl so that no more chromosin can be removed in this way, it is found that still more desoxyribose nucleoprotein can be extracted by desoxycholate.

A possible disadvantage of desoxycholate is that, being a detergent, it is far more likely than M NaCl to alter proteins, for it is known that detergents readily denature proteins. But this would perhaps appear to be a doubtful disadvantage because the purpose of Avery and his colleagues was not to prepare a nucleoprotein. They proceeded on the contrary to strip from the extracted nucleic acid as much protein as they could, for they found that the apparently protein-free material was still active as a transforming agent. This would mean that when pneumococcus chromosin acts as a transforming agent, its protein components are superfluous.

Avery and his colleagues have shown decisively by inactivation experiments that desoxyribose nucleic acid is an essential part of the transforming agent, and if there actually is no protein in their preparation, it would be obvious that the agent consists of nothing but nucleic acid. This is a conclusion of the greatest interest in the study of the chemical basis of biological specificity, and it should therefore be scrutinized carefully. There can be little doubt in the mind of anyone who has prepared nucleic acid that traces of protein proba-

bly remain in even the best preparations. With the tests now available for detecting how much protein is present in a nucleic acid preparation, it is probable that as much as 1 or 2 per cent of protein could be present in a preparation of "pure, protein-free" nucleic acid. One of the most sensitive direct tests for protein is the Millon reaction, but in our experience a nucleic acid preparation containing as much as 5 per cent of protein would give a negative Millon test. At present the best criterion for the purity of a nucleic acid preparation is its elementary composition and especially the nitrogen:phosphorus ratio. Presence of 2 per cent of protein would increase this ratio, but only by an amount that is well within the range of variation found for the purest nucleic acid preparations. No experiment has yet been done which permits one to decide whether this much protein actually is present in the purified transforming agent and, if so, whether it is essential for its activity; in other words, it is not yet known which the transforming agent is—a nucleic acid or a nucleoprotein. To claim more, would be going beyond the experimental evidence.

For further investigation of the transforming agent it may be advantageous to begin with pneumococcus chromosin, strip off its protein components step by step, and at the same time assay the material for transforming activity. If protein, as well as nucleic acid, is an essential component of the transforming agent, it is probable that this protein is only one of the two different protein fractions of chromosin.[6]

Dissociation of Histone and Nucleic Acid in Chromosins.—It is generally supposed that histone and nucleic acid are combined by salt-like linkages. The extent to which these linkages are dissociated in 1 M NaCl is not known. That they are at least somewhat dissociated in the chromosin (a nucleoprotamine) of trout sperm was shown by dialyzing this chromosin while dissolved in 1 M NaCl. As a result of prolonged dialysis all of the protamine diffused through the cellophane membrane leaving pure nucleic acid within the membrane. This experiment cannot be carried out for a histone-containing chromosin because histone molecules are too large to pass through a cellophane membrane. Histone molecules are, however, probably much smaller than the particles of highly polymerized desoxyribose nucleic acid, and it therefore seemed that by centrifuging at high speed a chromosin dissolved in M NaCl and observing the rates of sedimentation of histone and nucleic acid information would be obtained of the degree of dissociation of these two components in a chromosin. High speed centrifugation of thymus chromosin dissolved in 1 M NaCl does in fact show that histone sediments far more slowly than does the nucleic acid, and that there is much dissociation.

[6] The preliminary experiments that we have done on pneumococcus chromosin show that the histone fraction constitutes a little more than 50 per cent of the total chromosin and the non-histone protein only a few per cent.

Sedimentation was carried out in an air-driven centrifuge running at 35,000 R.P.M. The centrifuge holds 14 tubes, each with a capacity of 7.5 ml. Before being placed in the centrifuge the head was cooled down to 3–4°C. and then loaded with the cold chromosin solution. Since the centrifuge head turns in a vacuum, even after a run of 5 hours, the chromosin solution was still cool. The centrifuge tubes were removed as gently as possible and then, beginning at the top of each tube, 1 ml. samples were removed from each tube and mixed together. This is layer I. Layer II consists of the next 2 ml. from each tube and layer III of a still lower 2 ml. At the very bottom of the tube is a small, dense, gelatinous pellet.[7] Nitrogen and phosphorus analyses were done on each of the three layers.

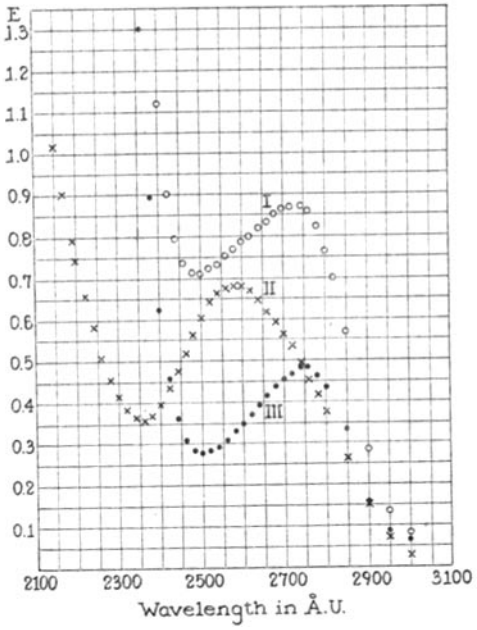
TABLE II

1	2	3	4
Material	N	P	N/P
	mg. per ml.	*mg. per ml.*	
Original chromosin.....	0.670	0.1880	3.57
Layer I.....	0.270	0.0045	60.00
Layer II.....	0.340	0.0328	10.3
Layer III.....	0.492	0.0918	5.36

The results of these analyses are given in Table II. The different nitrogen-phosphorus ratios at the various levels demonstrate that as sedimentation proceeds the composition changes. Since the ratio of nitrogen to phosphorus is higher in the upper layers it means that nucleic acid sediments more rapidly than one fraction, at least of the protein. The difference in composition of the original chromosin and of the material in the top layer after centrifuging can be perceived at once when ultraviolet absorption spectra of them are compared (Text-fig. 2). The curve of the material of the top layer is essentially that of a protein with a slight admixture of nucleic acid while the curve of chromosin is that of nucleic acid itself as far down as 2450 A. u., when the presence of protein becomes perceptible. The material in the top layer is dialyzed to remove the salt present. At the same time a slight precipitate forms and this is removed by centrifuging. In the precipitate is the trace of nucleic acid that was detected both by phosphorus analysis and spectrophotometrically. The material

[7] The composition of the pellet will be described in another paper. This pellet contains protein, some of which is histone, that is not dissociated from its combination with nucleic acid by the presence of concentrated salt. It also contains formed bodies that represent the residual structure of the chromosomes after removal of most of their protein and nucleic acid with 1 M NaCl. Presence of these "residual chromosomes" shows that while M NaCl dissolves much of the chromosome material, their residual structure material becomes merely dispersed in the viscous solution.

in solution now gives a typical protein ultraviolet absorption curve. None of the protein in this solution is precipitable by the $HgSO_4$—H_2SO_4 reagent. It is, however, precipitated by addition of ammonium hydroxide and does not redissolve in an excess of this reagent. The protein of the top layer which is dissociated from nucleic acid consists, therefore, of histone and nothing but histone.



TEXT-FIG. 2. Ultraviolet absorption curves of: I, the top layer of a thymus chromosin solution after centrifugation at 35,000 R.P.M.; II, the chromosin before being centrifuged, diluted 1 part in 60; III, the histone solution prepared by dialyzing the top layer.

Properties of Chromosins.—The properties of chromosins when dissolved in 1 M NaCl are readily explained by the high degree of dissociation of histone and nucleic acid in 1 M NaCl. The significance of dissociation will be better understood if it is first explained that when a chromosin is in 0.14 M NaCl (physiological saline) or in 0.02 M NaCl its properties are quite different from what they are in M NaCl, and in both 0.14 M and 0.02 M NaCl a chromosin is only slightly, if at all, dissociated. All chromosins are insoluble in physiological saline; some are soluble in 0.02 M NaCl.

The insolubility of chromosins in 0.14 M NaCl is sufficient evidence that in this medium histone and nucleic acid are combined, for both histone and nucleic acid are soluble in neutral 0.14 M saline when present alone. The fibrous char-

acter of the precipitate that occurs in 0.14 M NaCl appears to be due to the nucleic acid part of the complex, for when protein-free nucleic acid is precipitated in alcohol the precipitate is as beautifully thread-like as that of a chromosin in 0.14 M NaCl.

A chromosin precipitated in 0.14 M saline is fibrous because its nucleic acid component is highly polymerized. The very fact that the chromosin is insoluble in 0.14 M saline is also due to the highly polymerized state of its nucleic acid, for if a fibrous chromosin precipitate in saline is treated with an enzyme that depolymerizes desoxyribose nucleic acid, the chromosin loses its fibrous character and at the same time about two-thirds of it passes into solution. In an experiment on thymus chromosin 30 ml. of a fibrous suspension were treated with 15 mg. of the depolymerase prepared by McCarty's method (25). After 1 hour at 30° no fibers were any longer present. A portion of the suspension was centrifuged. Nitrogen and phosphorus analyses were done on the original suspension and on the clear supernatant obtained after centrifuging. The suspension contained 9.56 mg. of nitrogen and 2.66 mg. of phosphorus per ml., whereas the supernatant was found to contain 6.18 mg. of nitrogen and 2.10 mg. of phosphorus per ml.

The combination of histone and highly polymerized nucleic acid to form a solid fibrous structure in the biological range of pH and salt concentration is due to the basic character of histones. The special properties of histones in this respect become apparent when they are compared with proteins such as egg albumin, serum albumin, and serum globulin, which have isoelectric points on the acid side of neutrality. These proteins do not form insoluble precipitates with nucleic acid in physiological saline with a broad pH range near neutrality. For egg albumin and serum albumin the pH must be dropped below 5.0 and for serum globulin below pH 5.7 before a precipitate with nucleic acid forms. Histones, on the contrary, form precipitates with nucleic acid in 0.14 M NaCl all the way up to pH 9.0 and 10.0. These precipitates form either when isolated histone is added to pure nucleic acid or when a chromosin in 1 M NaCl is diluted. In experiments with the chromosin prepared from chicken erythrocytes, for example, the M NaCl solution was diluted to 0.14 M saline in borate and glycine buffers at various pH at 0°. Above pH 10.3 no precipitate of chromosin formed, but up to pH 10.3 precipitates were obtained, and in them the nitrogen-phosphorus ratio of 3.7 to 3.8 (an indicator of the ratio of protein to nucleic acid in a chromosin) remained the same from pH 6.8 to 10.3. It is the insolubility of chromosins in physiological saline that makes possible the thorough washing of a tissue with saline at neutrality or even at pH 9.2 before extraction with M NaCl. Above pH 10 chromosins are soluble because a point is reached where histones no longer act as bases and where, consequently, dissociation of histone and nucleic acid takes place. And chromosins are soluble in M NaCl because even at neutrality the linkage between histone (or protamine) and nucleic acid is broken by the concentrated salt.

A chromosin is soluble in M NaCl and insoluble in 0.14 M NaCl. As the concentration of salt is decreased below 0.14 M the chromosin fibers swell and tend to dissolve. In 0.02 M NaCl many chromosins are quite soluble. A convenient way to dissolve a chromosin in 0.02 M NaCl is to place a 1 M NaCl solution of the chromosin in a cellophane tube and dialyze the contents against distilled water until practically all the salt has passed out of the membrane. In the course of dialysis the chromosin precipitates, swells, and to some extent dissolves. Separation of swollen from dissolved material is facilitated if a little salt is present, and enough sodium chloride is, accordingly, added to the dialyzed chromosin to bring the salt concentration to 0.02 M. The mixture is then centrifuged for an hour at 10,000 R.P.M. The quantity of chromosin remaining in the supernatant varies considerably. If the chromosin used was prepared from the gray matter of beef brain, no material remains in solution. Practically all of the chromosin prepared from sheep spleen, on the other hand, remains in solution under these conditions. These are the limits between which other chromosins fall.

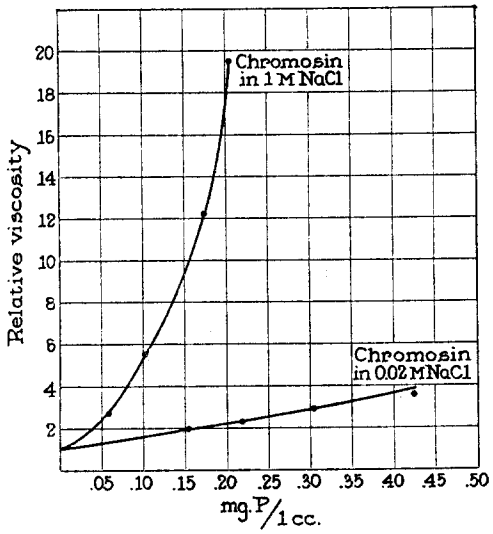
It has been shown that the histone and nucleic acid of a chromosin are markedly dissociated when the chromosin is dissolved in M NaCl and that they are combined when the chromosin is an insoluble precipitate in 0.14 M NaCl. It will now be seen that nucleic acid and histone are also combined when a chromosin is dissolved in 0.02 M NaCl. Experiments on the electrophoresis of a chromosin dissolved in 0.02 M salt show this clearly. In a 0.02 M, pH 7.46 veronal buffer the chromosin made from liver migrates towards the anode as one component. More extensive experiments along these lines have been done by Hall (26) on "thymus nucleohistone."

That the histone and nucleic acid of a chromosin in 0.02 M salt are combined is also shown by a study of chromosin films. Chromosin spreads at an air-water interface and in the film nucleic acid and histone are combined. Spleen chromosin dissolved in 0.02 M NaCl and mixed with a little heptyl alcohol spreads on a solution containing 0.01 M, pH 7.3 phosphate buffer. After 8 minutes the film is 13 to 14 A. u. thick. If the film is left in the tray overnight, its thickness is found not to have changed. Chromosin also spreads when the underlying solution is a 0.02 M, pH 4.04 acetate buffer. The film is 15 to 16 A. u. thick. Since isolated nucleic acid does not spread, and since histone spreads (at pH 7.3) to give a film 8 to 9 A. u. thick, the thickness characteristic of other proteins, the size of the films formed by chromosin indicates that in these films nucleic acid and histone are combined.[8]

The significance of dissociation is shown clearly by a comparison of the properties of chromosin in 1 M NaCl and in 0.02 M NaCl. The first property of a chromosin encountered while it is being isolated is its exceedingly high

[8] The electrophoresis and film spreading experiments were kindly done for us by Dr. Longsworth and Dr. Rothen.

viscosity when extracted in M NaCl. The loss of viscosity that occurs when the chromosin is dissolved in 0.02 M NaCl is at once apparent. This impression is borne out by viscosity measurements made in an Ostwald viscosimeter and shown in Text-fig. 3. The high viscosity of chromosin in M NaCl is apparently due to the dissociated nucleic acid. The protein hardly contributes to the viscosity, for when most of the protein in the chromosin is removed the viscosity is unaffected. Removal of protein is accomplished by shaking the



TEXT-FIG. 3. Curves showing the viscosities of chromosin dissolved in 1 M NaCl and in 0.02 M NaCl. The concentrations, given in the abscissa are recorded as milligrams of nucleic acid phosphorus per milliliter, the nucleic acid concentration being 10 times this figure.

chromosin dissolved in M NaCl with a chloroform-octyl alcohol mixture. Before shaking, the chromosin solution had a phosphorus content of 0.103 mg. per ml. and a nitrogen-phosphorus ratio of 3.9. After shaking, the phosphorus content was 0.104 mg. per ml. and the nitrogen-phosphorus ratio was 2.2, indicating that all of the nucleic acid remained in solution but that most of the protein had been removed. The relative viscosity was 5.5 both before and after the protein had been removed.

Highly polymerized desoxyribose nucleic acid is viscous and exhibits marked birefringence of flow. A chromosin dissolved in M NaCl also shows birefringence of flow, and this property, like the high viscosity, can be attributed to dissociated nucleic acid, for the solution of undissociated chromosin in 0.02 M NaCl does not show birefringence of flow. Nucleic acid solutions are birefrin-

gent when their particles are highly elongated. It may be concluded, then, that in an undissociated chromosin solution the chromosin particles are not markedly elongated.

Dissolving a chromosin in 0.02 M NaCl changes it so that when the material is again brought into M NaCl it is obviously different from a chromosin that has at no time been in 0.02 M NaCl. This change can be seen at once when sufficient salt is added to a 0.02 M NaCl solution of chromosin to bring the salt concentration to 0.14 M; the chromosin precipitates, but in a granular form or in the form of "moth-eaten" fibers, entirely different from the beautiful fibrous precipitate of an unaltered chromosin. The character of the precipitate is not restored by first adding to the solution in 0.02 M NaCl enough salt to bring the concentration to 1 M and then precipitating the chromosin by diluting the salt to 0.14 M. The low viscosity of chromosin dissolved in 0.02 M NaCl (low compared with the viscosity of the original chromosin in M NaCl) is increased considerably by raising the salt concentration to 1 M, and so dissociating nucleic acid from its combination with histone, but even so the viscosity remains far below its original level. A chromosin solution containing 0.43 mg. P per ml. and having a relative viscosity of 3.8 in 0.02 M NaCl has its relative viscosity increased to 6.1 by raising the salt concentration to 1 M. It can be seen from Text-fig. 3 that the viscosity of the original chromosin in 1 M NaCl is many times higher.

Since the viscosity of a chromosin solution in 1 M NaCl is due mainly to the high degree of polymerization of its nucleic acid, it would seem as if depolymerization occurs to some extent while a chromosin is in 0.02 M NaCl. An enzyme that depolymerizes desoxyribose nucleic acid is known to have a widespread distribution. It may be supposed that some of this enzyme is carried along with the chromosin solution, but that it is inactive in M NaCl (possibly because it cannot combine with nucleic acid in presence of so much salt) and becomes active only when the salt concentration is reduced. If enzymatic activity is responsible for the change in a chromosin that occurs in 0.02 M NaCl, the extent of this activity would appear to vary greatly from one preparation to another. We find, for example, in 3 different preparations of sheep spleen chromosin that the relative viscosities of a 0.02 M NaCl chromosin solution containing 0.2 mg. P per ml. are 1.2, 2.2, and 5.0. There are also marked differences in the chromosins isolated from different tissues. The chromosins prepared from trout sperm and beef brain do not dissolve at all in 0.02 M NaCl. Does this mean that the depolymerizing enzyme is not present in trout sperm cells or in the cells of the brain? And, if so, is the absence of this enzyme related to the fact that these cells are not destined to divide?

The changes known to occur in a chromosin when it is dissolved in 0.02 M NaCl enable us to understand the method for preparation of thymus nucleohistone and nucleic acid used by Huiskamp (7), Bang (*7a*), and Hammarsten (22).

In this method the minced thymus gland is immersed in a large volume of water, so that the final salt concentration is not far from 0.02 M. The suspension is allowed to stand for some 24 hours, during which time the nucleohistone gradually passes into solution. When the extracted nucleohistone is precipitated by bringing the salt concentration to 0.14 M, a non-fibrous granular mass forms. Apparently some depolymerization has occurred, but this process has certainly not gone far, for the nucleic acid finally prepared is viscous and fibrous when precipitated from alcohol. If it is true that nucleohistone dissolves in 0.02 M NaCl only because an incipient depolymerization of nucleic acid occurs, it may be inferred that the minced thymus tissue is suspended for so many hours in water to permit a certain degree of autolysis to take place. Our attempts to prepare "nucleohistone" from liver and kidney by extraction with water have failed. This may be because in these tissues autolysis proceeds too rapidly. Aqueous extraction of nucleohistone from brain tissue, on the other hand, would probably fail because depolymerization of the chromosin of this tissue does not occur.

Extraction of Ribose Nucleoproteins.—The insolubility of chromosin in 0.14 M NaCl makes it possible to wash a tissue thoroughly with saline, and in the washings extract ribose nucleoproteins without dislodging the desoxyribose nucleoproteins. In this way even from a tissue like the thymus, which contains 15 times as much desoxyribose as ribose nucleic acid, the latter can be extracted with only an exceedingly slight admixture of the former. We have prepared ribose nucleoproteins from calf thymus, calf liver, and wheat germ by the same method and with essentially the same results. Only the product prepared from thymus will be described.

It is a curious fact that 52 years ago in the same paper in which Lilienfeld (27) reported his discovery of thymus nucleohistone, he noted that the thymus also contains another nucleoprotein with far lower phosphorus content. This nucleoprotein was also described by Huiskamp (7) in his important study of thymus nucleohistone. Huiskamp found that it contained 0.96 per cent phosphorus. At the time of Lilienfeld's and Huiskamp's work the distinction between desoxyribose and ribose nucleic acids was not yet made. The thymus nucleoprotein of low phosphorus content prepared by Lilienfeld and Huiskamp, it will presently be shown, is a ribose nucleoprotein.

Although our method of preparing this nucleoprotein differs somewhat from Huiskamp's there can be little doubt that essentially the same substance is prepared by the two procedures. Huiskamp extracted the thymus with water. The aqueous extract contained both chromosin and the low phosphorus nucleoprotein. They were separated from each other by adding a little salt, which caused chromosin to precipitate, leaving in solution the low phosphorus nucleoprotein. The latter was then precipitated by acidifying with acetic acid. Our method depends upon the fact that chromosin is insoluble, but that the

low phosphorus nucleoprotein is soluble, in physiological saline, so that when the minced thymus tissue is extracted with 0.14 M NaCl, the extract contains the low phosphorus nucleoprotein, but no chromosin. Acidifying the extract with acetic acid precipitates the nucleoprotein and it is subsequently dissolved in sodium bicarbonate. The material is precipitated with acetic acid and dissolved in bicarbonate a number of times.

The final product contains 1.06 per cent phosphorus and 15.3 per cent nitrogen. When tested with the diphenylamine reagent, not even a trace of desoxyribose nucleic acid can be detected. The presence of ribose nucleic acid, however, can be demonstrated. For this purpose it is necessary to remove the protein, and this is done by shaking the nucleoprotein with a chloroform-octyl alcohol mixture. The material remaining in solution is ribose nucleic acid, for its absorption spectrum in the ultraviolet shows the characteristic nucleic acid curve and the ratio of pentose to phosphorus is precisely the same as it is in a sample of pure ribose nucleic acid prepared from yeast.

The thymus nucleoprotein of low phosphorus content was recently prepared by Mazia (28), following the method used by Huiskamp. He found that the preparation gives a positive Feulgen reaction (indicating the presence of desoxyribose) and for this reason he decided that it is derived from the chromatin of the cell nucleus. With a sensitive spot test for desoxyribose, like the Feulgen reaction, it is impossible to distinguish between two possibilities: that the material being tested contains a trace of desoxyribose; or that in the material being tested all of the nucleic acid is of the desoxyribose type. Since the thymus nucleoprotein of low phosphorus content has now been identified as a ribose nucleoprotein, it would seem probable that the desoxyribose responsible for the positive Feulgen test in Mazia's preparation was derived from a relatively small quantity of chromatin material that became mixed with the mass of ribose nucleoprotein. Such an admixture could easily happen in Huiskamp's procedure; and it would also occur in our procedure, for we have observed that even in the cold an exceedingly rapid autolysis takes place in the cell nucleus leading to the formation of small quantities of desoxyribose nucleoprotein soluble in physiological saline.

To prepare thymus ribose nucleoprotein 350 gm. of calf thymus are cut into small pieces with scissors and then, suspended in 1600 ml. of 0.14 M NaCl, minced in a high speed electric mixer. The mixture is stirred for several hours and centrifuged. To the slightly opalescent supernatant 10 per cent acetic acid is added until a copious precipitate appears, pH approximately 4.2. On centrifuging a white precipitate is obtained. This is well washed with 0.5 M NaCl and the salt is then removed by washing with water. While the protein suspended in water is stirred, 0.5 M $NaHCO_3$ is slowly added until most of the material dissolves. The volume of the mixture now is about 500 ml. It is centrifuged at 10,000 R.P.M. and a slightly opalescent supernatant is obtained. The protein in solution gives an intense test for SH groups when

nitroprusside and ammonium hydroxide are added. The protein is twice more precipitated with acetic acid and dissolved in bicarbonate. The final solution is dialyzed free of salt and then centrifuged, discarding the precipitate. A small sample of this solution is taken for determination of its ultraviolet absorption spectrum. A larger part is acidified and the precipitated material is thoroughly extracted with hot alcohol, ether, and dried at 106°. The dried material is analyzed for phosphorus and nitrogen. The remainder of the solution is shaken with a chloroform-octyl alcohol mixture. After prolonged shaking, the aqueous phase is separated. It contains 0.0488 mg. P and 0.109 mg. N per ml., a N:P ratio of 2.24. Since the N:P ratio of the solution was originally 11.0, it can be seen that most of the protein has been separated from nucleic acid. The solution with the N:P ratio of 2.24 was used for pentose estimations and for determination of the absorption spectrum. For the test with diphenylamine a quantity containing 0.141 mg. P was used and no blue color was obtained. Since a quantity of desoxyribose containing less than one-seventh of this amount of phosphorus would have given an intense blue color, it is obvious that no more than traces of desoxyribose nucleic acid can be present. The absorption coefficient at 2600 A. u. and pentose estimations show that all of the phosphorus can be accounted for as ribose nucleic acid.

In the preparation of a chromosin its separation from ribose nucleoproteins depends upon the fact that the latter are soluble and chromosin is insoluble in neutral, physiological saline. And, in somewhat the same way, the older workers separated nucleohistone from the nucleoprotein of low phosphorus content (which is now known to be a ribose nucleoprotein) by depending upon the same difference in solubility in dilute neutral salt solutions. Recently Greenstein and Jenrette (29) have prepared "liver nucleoprotein" by a procedure in which apparently no step is taken to separate the two kinds of nucleoprotein. Minced liver is extracted with 0.5 M KCl and a little bicarbonate to bring the pH to 8.0. The extracted material is precipitated at pH 4.2 and then redissolved at pH 10.5. This procedure is repeated 6 times. In the original paper by Greenstein and Jenrette nothing is said about the location within the cell of "liver nucleoprotein." Recently in a general review of nucleoproteins Greenstein (30) in referring to his preparation of liver nucleoprotein, says, "the presence of desoxyribose nucleic acid in such complexes strongly suggests the nuclear origin of the latter."

In the procedure of Greenstein and Jenrette nothing is done to separate desoxyribose from ribose nucleoproteins. Both kinds of nucleoprotein would be extracted by their procedure, both kinds would be precipitated from the extraction fluid, both kinds would be redissolved, and so forth until at the end a mixture of both kinds should be present. A test for desoxyribose showed that this substance was present in the final product, but its presence does not mean that ribose was not present. A quantitative determination of desoxyribose would be far more significant, and there is for this purpose the excellent diphenylamine method of Dische. We have prepared "liver nucleoprotein"

following the directions of Greenstein and Jenrette, and we have determined its desoxyribose content by Dische's method.

The product we prepared had, after removal of any lipid present, a phosphorus content of 0.83 per cent, close to the 0.81 per cent found by Greenstein and Jenrette. In the presence of as much protein as this phosphorus content would indicate, the Dische method tends to give values for desoxyribose that are somewhat high. For the diphenylamine reaction 2 ml. of a nucleoprotein suspension were used to which 1 ml. of water and 6 ml. of reagent were added. The 2 ml. contained 14.0 mg. of nucleoprotein, equivalent to 0.116 mg. of phosphorus. As a standard a sample of pure desoxyribose nucleic acid containing 0.025 mg. phosphorus was used. After the mixtures were heated those which contained liver nucleoprotein were turbid and were therefore centrifuged, giving a perfectly clear supernatant and a colorless precipitate. The color of the supernatant was a blue-green and did not match the blue color of the standard. The absorption spectrum of the standard has a maximum at 6000 A. u.; and extinction coefficients were measured at this wave-length. Extinction coefficients of 0.180 for the standard and 0.227 for the liver nucleoprotein were obtained. This shows that at the most 27.2 per cent of the total phosphorus in liver nucleoprotein is "desoxyribose nucleic acid phosphorus." Davidson and Waymouth (31) found that in sheep liver the ratio of ribose nucleic acid phosphorus to desoxyribose nucleic acid phosphorus is 3.5 to 1, or that the desoxyribose phosphorus is 22.6 per cent of the total nucleic acid phosphorus. This is close to the value of 27.2 per cent we find for the part of the total liver nucleoprotein phosphorus which according to the diphenylamine reaction is in the form of desoxyribose nucleic acid. It would seem then, that the liver nucleoprotein of Greenstein and Jenrette consists of a general mixture of desoxyribose and ribose nucleoprotein, and this is just what would be expected from the method of preparation. It may be added that we have carried out experiments in which we treated liver nucleoprotein with Kunitz's ribonuclease and found that much phosphorus was thereby removed from the nucleoprotein.

SUMMARY

A desoxyribose nucleoprotein complex, which we have referred to as a *chromosin*, has been prepared from a great variety of cells, mainly animal but also plant and bacterial. A chromosin is derived from the cell nucleus. In the course of preparation precautions have been taken to prevent contamination by cytoplasmic constituents. To assure the nuclear origin of all components of chromosin, nuclei have in several instances been isolated before extraction was begun. Because of the precautions taken, chromosins do not contain detectable quantities of ribose nucleoproteins; but, incidentally, extraction of

ribose nucleoproteins, free of desoxyribose compounds, has also been described in this paper.

A typical chromosin contains 3 components: desoxyribose nucleic acid, histone, and non-histone protein. The nucleic acid, being highly polymerized, is exceedingly viscous when dissolved and fibrous when precipitated. Histone and non-histone protein differ from each other in a number of ways, of which one of the most definite is that whereas a histone contains no more than traces of tryptophane, the non-histone protein of chromosin contains nearly 1 per cent of tryptophane. In neutral physiological saline both proteins can combine with nucleic acid. With the isolation of chromosins from so many different kinds of cells, it can now be seen that (contrary to the view expressed by Kossel) histones are present in most animal cells and at least in some plant and bacterial cells.

Chromosin prepared from the Type III pneumococcus is active in transforming the type of a pneumococcus culture. It has been pointed out that it is not yet known whether or not protein is a necessary constituent of the transforming agent.

To extract chromosin from a cell M NaCl is used. When dissolved in M NaCl the nucleic acid and histone components of a chromosin are to a considerable extent dissociated. They are not dissociated when the chromosin is dissolved in 0.02 M NaCl, but in this medium a partial depolymerization of the nucleic acid occurs.

A chromosin should certainly not be considered to be a definite chemical compound. It is a complex extracted from chromatin, which is itself a complicated nuclear structure. And in the course of extraction, it need hardly be said, the structure of chromatin has been considerably changed. To avoid complications it has been considered an advantage in this work to begin with isolated nuclei, and it would clearly be a further simplification to begin chemical procedures only after the chromosomes themselves have been isolated. This is now being accomplished, and it is found that the methods described in this paper are of value in learning how the substances present in a chromosin are put together in a chromosome.

REFERENCES

1. Mirsky, A. E., and Pollister, A. W., *Proc. Nat. Acad. Sc.*, 1942, **28**, 344.
2. Pollister, A. W., and Mirsky, A. E., *J. Gen. Physiol.*, 1946, **30**, 101.
3. Kossel, A., The Protamines and Histones, New York, Longmans, Green and Co., 1928.
4. Caspersson, T., *Chromosoma*, 1940, **1**, 562.
5. Steudel, H., *Z. physiol. Chem.*, 1914, **90**, 291.
6. Stedman, E., and Stedman, E., *Nature*, 1943, **152**, 267.
7. Huiskamp, W., *Z. physiol. Chem.*, 1901, **32**, 145.

7*a*. Bang, I., *Beitr. chem. Physiol. u. Path.*, 1903, **4**, 115, 331.

8. Miescher, F., in Hoppe-Seyler, F., Medizinisch-chemische Untersuchungen, Berlin, A. Hirschwald, 1871, **4**, 441.

9. Bensley, R. R., *Anat. Rec.*, 1938, **72,** 351.
10. Banga, I., and Szent-Györgyi, A., *Enzymologia*, 1941, **9,** 111.
11. Avery, O. T., MacLeod, C. M., and McCarty, M., *J. Exp. Med.*, 1944, **79,** 137.
12. Mirsky, A. E., Ris, H., and Pollister, A. W., unpublished experiments.
13. Kaskowski, M., and Ryerson, D. C., *Arch. Biochem.*, 1943, **3,** 227.
14. Crossmon, G., *Science*, 1937, **85,** 250.
15. Stoneburg, C. A., *J. Biol. Chem.*, 1939, **129,** 189.
16. Marshak, A., *J. Gen. Physiol.*, 1941, **25,** 275.
17. Dounce, A. L., *J. Biol. Chem.*, 1943, **147,** 685.
18. Allen, R. J. L., *Biochem. J.*, 1940, **34,** 858.
19. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46,** 2066.
20. Dische, Z., *Mikrochemie*, 1930, **8,** 4.
21. Sevag, M. G., Lackman, D. B., and Smolens, J., *J. Biol. Chem.*, 1938, **124,** 425.
22. Hammarsten, E., *Biochem. Z.*, 1924, **144,** 383.
23. Shaw, J. L. D., and McFarlane, W. D., *Canad. J. Research, Sect. B*, 1938, **16,** 361.
24. Alloway, J. L., *J. Exp. Med.*, 1933, **57,** 265.
25. McCarty, M., *J. Gen. Physiol.*, 1946, **29,** 123.
26. Hall, J. L., *J. Am. Chem. Soc.*, 1941, **63,** 794.
27. Lilienfeld, L., *Z. physiol. Chem.*, 1894, **18,** 473.
28. Mazia, D., in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1941, **9,** 40.
29. Greenstein, J. P., and Jenrette, W. V., *J. Nat. Cancer Inst.*, 1940, **1,** 91.
30. Greenstein, J. P., Advances in Protein Chemistry, New York, Academic Press, 1944, **1,** 210.
31. Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1944, **38,** 39.

EXPLANATION OF PLATE 3

PLATE 3

FIG. 1. Isolated liver nuclei, unstained. × 2600.

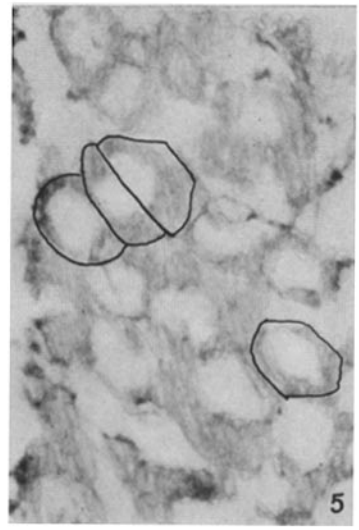
FIG. 2. Isolated thymus nuclei, unstained. × 2600.

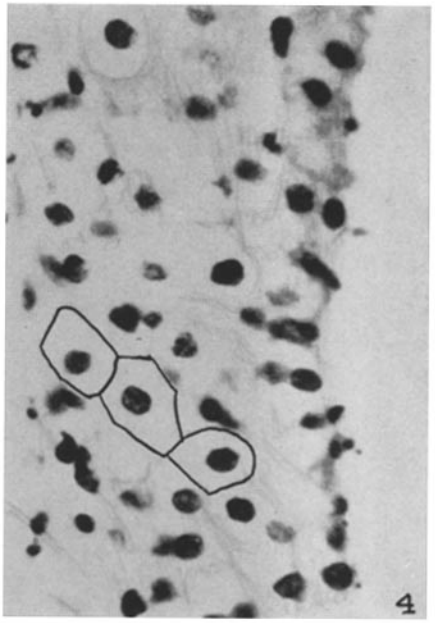
FIGS. 3, 4, and 5. These figures are from calf liver sections cut fresh at 100 microns on a freezing microtome, treated with salt solutions, and fixed in Zenker's fluid, then cut in paraffin at 6 microns and stained with hematoxylin and eosin. Photographed with a Zeiss microscope lamp and a blue-green filter (Jena glass BG 7). Negatives were given the same exposure, developed simultaneously, with the same exposure during enlargement and prints were developed together. Outlines of several cells have been emphasized by inked lines.

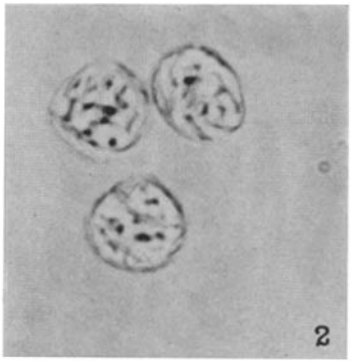
FIG. 3. Slice fixed immediately after cutting. Heavy stain in the cytoplasm indicates little loss of protein from the cells. × 1050.

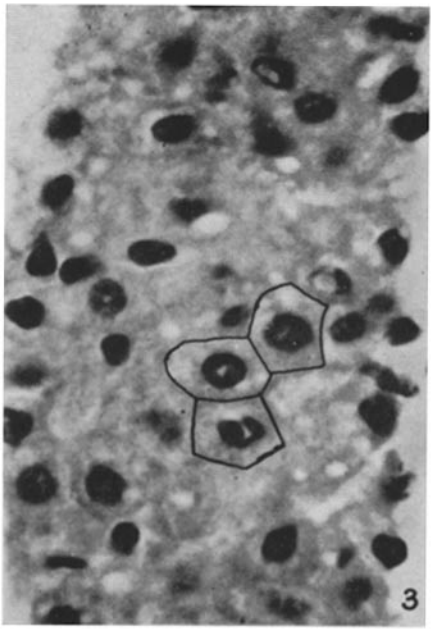
FIG. 4. Slice fixed after 3 hours' immersion in 0.14 M NaCl at 5°C. This has extracted a large part of the stainable material from the cytoplasm. × 1050.

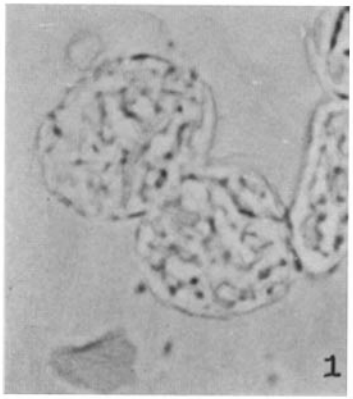
FIG. 5. Slice fixed after 1 hour in 1.0 M NaCl at 5°C. Nuclei are represented by spaces from which stainable material has been removed. × 1050.











(Mirsky and Pollister: Chromosin)