THE COMPOSITION AND STRUCTURE OF ISOLATED CHROMOSOMES

By A. E. MIRSKY and H. RIS*

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 2

(Received for publication, August 4, 1950)

Isolation of chromosomes from lymphocytes of the thymus and from erythrocytes of fish has been described (1). For further investigation of the chemistry of chromosomes, those isolated from other types of cells are needed. In this paper a procedure is given for isolating the chromosomes from mammalian liver, kidney, and pancreas. Their composition and structure have been investigated. These chromosomes have also been studied microscopically, and from this study has come evidence in addition to that previously presented that these chromatin-containing bodies are, in fact, chromosomes.

When the procedure which yields clean isolated chromosomes of the thymus is applied to liver, kidney, or pancreas, the chromosomes obtained are contaminated with cell debris. If such preparations are stained with aceto-orcein or with the Feulgen reagent, it is true that they appear fairly satisfactory; but when they are then counterstained with fast green, it is disappointing to see how much non-chromosomal material they contain. This material can be removed with dilute citric acid in the absence of salt. (Such treatment produces no apparent change in the composition of thymus chromosomes.) Citric acid is used reluctantly because it is sufficiently acid to denature some proteins; but unfortunately no other way of removing the contaminating material has been found.

Before coming to the special measures required to clean up liver, kidney, and pancreas chromosomes, a detailed description will be given of procedures now used in this laboratory for isolation of chromosomes from erythrocytes and thymus lymphocytes. All operations are carried out in a cold room maintained at 1 or 2° above zero. If a low temperature is not maintained, autolysis occurs and well formed chromosomes are not obtained.

1. Erythrocyte Chromosomes.—The erythrocytes from which chromosomes are prepared are those of the carp and the green turtle. The citrated blood is strain d and the erythrocytes are washed with saline. The suspension is then passed through a colloid mill several times. This fragments both cells and nuclei. On passing through

* Present address: Department of Zoology, University of Wisconsin, Madison.

the mill the fluid warms up several degrees and it is therefore collected in a flask immersed in an ice mixture. When the fragmented material is centrifuged at slow speed, a colorless sediment and clear red supernatant are obtained. The sediment, which consists of chromosomes, is washed with saline until colorless. Washing is accomplished by suspending the chromosomes in saline, centrifuging, and discarding the supernatant. Centrifuging is at low speed, just fast enough to cause sedimentation of chromosomes, but not fast enough to pack them together. When stirring the suspended chromosomes it is important that the stirring rod be perfectly centered so that movement can be rapid but not turbulent enough to cause the chromosomes to be beaten together into fibrous masses. A well centered stirrer can be run at high speed (5,000 to 12,000 R.P.M.) so that only very brief stirring is required.

In attempting to prepare chromosomes from fowl erythrocytes, it was found that the cells are broken but their nuclei remain intact after treatment that readily fragments the nuclei of carp and turtle erythrocytes. Prolonged action of the Waring blendor or colloid mill on chicken erythrocyte nuclei draws them out into long fibers, far longer than the original diameter of the nuclei. In a previous paper it has already been stated that preparations from chicken erythrocytes have not been as satisfactory as those from fish erythrocytes (1).

2. Thymus Lymphocyte Chromosomes.—About 150 gm. of ice cold fresh calf thymus are finely minced with scissors and then placed in a Waring belendor with 150 gm. of crushed ice and 350 ml. saline-citrate (sodium chloride concentration 0.14 M and sodium citrate 0.01 M), the citrate being added to inhibit desoxyribonuclease. The blendor is run at top speed for 6 minutes. For this length of time enough ice is present to keep the mixture at 0°C. The mixture is then strained. This is most readily accomplished by using first a twofold layer, then a fourfold layer of surgical gauze, next a single thickness of the finest towel, and finally a double thickness twice. The strained fluid is centrifuged at 2,200 R.P.M. for 15 minutes and the opalescent supernatant is discarded. The sediment is suspended in 500 ml. saline-citrate, stirred, and centrifuged at slow speed. After resuspending in saline-citrate the material is passed through the colloid mill and then centrifuged. The chromosomes should be washed twice more. As in the case of erythrocyte chromosomes, almost no intact nuclei remain among the chromosomes.

3. Chromosomes of Liver, Kidney, and Pancreas.—The first part of this preparation is much the same as for thymus chromosomes. Because of the smaller fraction of nuclear material in these tissues, three times as much tissue is taken, three blendors being used simultaneously. Our centrifuge is large enough to hold all of the strained fluid, about 1,700 ml. To sediment the chromosomes of these tissues the centrifuge must be run somewhat faster than was necessary for thymus chromosomes. The sediment suspended in a volume of 1,700 ml. saline-citrate is washed twice before being passed through the colloid mill and twice thereafter. It is then suspended in 1,700 ml. of 0.2 per cent citric acid and centrifuged at low speed. In subsequent centrifuging it is necessary to increase the speed somewhat because as salt is removed from the citric acid suspension the chromosomes sediment less rapidly. The sediment is washed twice with 1,700 ml. of 0.2 per cent citric acid and then passed through the mill again. This breaks up clumps, removing much of the material holding masses of chromosomes together. The chromosomes are washed twice more and finally brought to a volume

of 1 liter, stirred, and allowed to stand overnight in a tall beaker. In the morning the material has settled into two layers, the upper and by far the larger of which is decanted and kept, the lower being discarded. The chromosomes are washed with 1,700 ml. of 0.2 per cent citric acid and then brought to a volume of 140 ml. This contains about 1.4 gm. of chromosomes, dry weight. Very few intact nuclei are present.

Satisfactory chromosome preparations have been made from liver, kidney, and pancreas of cattle, sheep, and horses, and from the pancreas of domestic fowl. Preparations made from liver and kidney of rats and rabbits contained well formed chromosomes, but were grossly contaminated with non-chromosomal material. Preparations made from swine organs are also contaminated but this may be due to the fact that the animals were scalded at the slaughter house.

Tests for Contamination by Non-Chromosomal Material.—A sensitive test for particulate contamination is to stain the material with a mixture of orcein and fast green. Stained in this way, thymus chromosomes take orcein, becoming red, and practically no green-staining material can be seen. Liver, kidney, and pancreas chromosomes before citric acid treatment can be seen to be contaminated with much green-staining material, but this is removed by citric acid so that little or no green-staining contamination is visible.

Staining with orcein–fast green would not necessarily detect contaminating substances that adhere to the chromosomes, and it might be supposed that chromosomes from cells with abundant cytoplasm would be more likely to be contaminated in this way than would thymus chromosomes since thymus lymphocytes have a relatively scanty cytoplasm. If this were the case, it might even be that the lower percentage of desoxyribonucleic acid found in isolated liver, kidney, and pancreas chromosomes (26 to 28 per cent as compared with 39 per cent in thymus chromosomes) is simply due to the large quantities of cytoplasmic substances adhering to these chromosomes. The following experiment, in which thymus chromosomes were isolated from a medium containing kidney cytoplasm, was done to test this possibility.

Chromosomes were isolated from calf kidney and thymus minced together, using 360 gm. of the former and 72 gm. of the latter. With these masses of tissue the kidney accounted for about 90 per cent of the cytoplasmic material, and approximately equal masses of thymus and kidney chromosomes were present. From the mixed tissues chromosomes were isolated by the procedure ordinarily followed for kidney chromosomes. The final suspension in citric acid contained 1,570 gm. of chromosomes which, when examined microscopically, were clean and well formed. From the desoxyribonucleic acid content of the chromosomes it can be seen whether kidney cytoplasmic substances have adhered to the chromosomes—in which event they would now all have the relatively low nucleic acid content characteristic of chromosomes isolated from the kidney (27.5 per cent)—or whether no constituents of kidney cyto-

plasm adhere to the chromosomes so that the nucleic acid content of the mixed chromosomes lies between the values characteristic for those isolated from the kidney and thymus. The desoxyribonucleic acid content of the mixed chromosomes was found to be 32.8 per cent. This is close to the value to be expected, about 33 per cent, if no contamination of the thymus chromosomes occurred.

A study of the amino acid composition of proteins in isolated chromosomes is of value in deciding whether the relatively high protein content of chromosomes isolated from the kidney, compared with those from the thymus, is due to contamination. The proteins of isolated chromosomes that remain after histones have been removed, the so called residual protein fraction, have been analyzed by the starch column chromatographic procedure of Moore and Stein, which gives as precise and complete an amino acid analysis as is now possible (2). The complete results of these analyses will be reported in another paper (3). Two characteristic differences in amino acid composition were found when the residual proteins of chromosomes were compared with mixed cytoplasmic proteins. Expressed in terms of grams of amino acid per 100 gm. of protein, it was found that in the residual proteins of all chromosomes investigated there was more glycine than serine and more arginine than lysine, whereas in the cytoplasmic proteins these ratios were reversed; that is, there was less glycine than serine and less arginine than lysine. These ratios do not warrant the conclusion that the residual protein of isolated kidney chromosomes was not at all contaminated by other protein, but they show that when four times as much residual protein is found in kidney chromosomes as in thymus chromosomes, most of this difference is not due to contamination.

Although gross contamination of isolated chromosomes sufficient to account for the differences in composition of preparations from kidney and thymus has not been found, it should be recognized that some contamination by protein probably occurs. The phosphoric acid groups of nucleic acid in a chromosome that are able to combine with a basic dye can also combine with a basic protein, and we have in fact found that isolated chromosomes can combine with extra histone. When cells are broken up, a basic cytoplasmic protein may adhere to the chromosomes. Washing of chromosomes with saline should remove the less basic proteins of the cell debris. If these proteins are not washed away, there is the danger that they will combine with phosphoric acid groups of nucleic acid when the chromosomes are suspended in citric acid.

Tests can be made for contaminants, but in the present experiments nothing has been done to determine what substances are removed from chromosomes by the solvents used in the isolation procedure. In all probability some soluble proteins which are loosely attached to the chromosomes are washed away. A study of nuclei which were isolated by means of non-aqueous solvents has shown how much water-soluble protein can be washed out of the nucleus (4). It is possible that some of this protein is loosely combined with chromosomes.

Microscopic Study. 1. Chromosomes from Various Tissues of Cattle.—The chromosomes isolated from interphase nuclei of the various tissues can be stained with orcein and studied cytologically. Thus it can be shown that these interphase chromosomes are visibly double, that they are helically coiled, and have characteristic longitudinal differentiation into thicker, more tightly coiled and thinner, more or less despiralized segments (1). Several well defined types of chromosomes can be recognized. They are characterized by their size and the relative lengths of thick and thin regions. A comparative study of chromosomes isolated from different tissues makes it possible to get some information on the interesting question whether the interphase chromosomes differ morphologically from one tissue to the next. The evidence obtained indicates that in mammalian tissue the chromosomes are morphologically alike, since it is possible to recognize several types of chromosomes in all the preparations from the various tissues. For instance, in Figs. 1 to 4 we see the same chromosome from liver, pancreas, kidney, and thymus, a chromosome of special interest since one can recognize clearly three gyres in the thick region. There can be little doubt about the identity of this chromosome.

Among the chromosomes prepared from interphase nuclei one often finds some which are still attached to a nucleolus. Through the work of Heitz (5) and McClintock (6) it is well known that nucleoli are formed at definite regions on certain chromosomes. Diploid nuclei in bovines have maximally 4 nucleoli which often fuse. One finds thus either 4 small, one medium and 2 small, 2 medium or 1 large nucleoli per nucleus. Nucleoli are therefore tags which mark certain chromosomes and since the same chromosome forms a nucleolus in the pancreas or liver for instance, this gives a sure means to identify a chromosome in different tissues. In preparations from liver and pancreas it was possible to identify one of the nucleolus-organizing chromosomes. Since the chromosome was never found entirely in one focal plane, a drawing has to show its morphology. The nucleolus is attached at one end of the chromosome to a thin segment between a trabant and the main body of the chromosome. At the opposite end is another thin region forming an angle with the thick part of the chromosome. Figs. 5 and 7 show this chromosome from liver and pancreas. The nucleolus-organizing chromosome therefore has the same morphological structure whether it is isolated from liver or from pancreas.

These observations provide additional evidence that the isolated chromatin-containing bodies are in fact chromosomes. In the classical work on chromosomes by Boveri (7) he laid great emphasis on the individuality of the chromosome and the importance of this characteristic of a chromosome has been recognized ever since. From this point of view there can be no doubt that there are chromosomes in our preparations. This is not to say that all the bodies in a preparation are chromosomes, for to establish this point it would be necessary to identify as an individual every thread-like particle and this has not been

done. A difficulty in identification is that most of the bodies are tangled together, sometimes two or three together and occasionally many more; another difficulty is that in a mammalian cell there is a large number of chromosomes, some of which differ only slightly from others. If the time is taken to study those bodies that are not entangled with others, in most cases it can be seen that they are fairly well formed chromosomes, not unlike the chromatin-containing bodies that are seen within the nuclei from which they were derived. It would be a mistake to suppose that a preparation of isolated chromosomes contains a few chromosomes in a mass of nondescript material.

2. Comparison of Mitotic Chromosomes and Chromosomes Isolated from Interphase Nuclei.—Makino described the mitotic chromosomes in domestic cattle, sheep, and swine (8). He found in cattle 30 pairs of chromosomes of various length, all rod-shaped. This means that the spindle attachment in all chromosomes is close to one end. Now in all forms where such rod-shaped chromosomes have been studied in detail it has been found that the spindle attachment is not at the very end, but that it is between a very small short arm and a large long arm of the chromosome. The short arm in these cases is usually very difficut to see. We may assume that the chromosomes of cattle also have a small short arm, though it is difficult to recognize during mitosis. Now it is characteristic of all chromosomes isolated from bovine tissues that they consist of a thick region and a thin segment at one end or both ends. It appears likely therefore that one or the other of these thin segments corresponds to the short arm which is largely despiralized in the interphase chromosome. Very often the two chromatids of this short arm are separated (Fig. 8).

3. Comparison of Chromosomes Isolated from Tissues of Cattle, Sheep, and Swine.—Chromosomes isolated from tissues of sheep and swine were compared with those of cattle. It was found that some of the characteristic types found in bovine tissues were also present in the preparations from sheep and swine. In Figs. 8 and 9 we see a chromosome from beef and swine. Though it is not surprising to find that related forms have some chromosomes which are similar morphologically this does not mean that they are identical also in detail. In addition to chromosomes similar to those from cattle one finds in preparations from sheep and swine tissues chromosomes which are quite different. It was mentioned above that the chromosomes from beef tissues consist of a thick region with thin, despiralized sections at one or both ends. In sheep and swine, however, one finds also interphase chromosomes which have a clear constriction between two thick regions (Figs. 10–13). Now Makino who studied the chromosomes of swine and sheep during mitosis found several V-shaped chromosomes at metaphase and anaphase. This contrasts with the situation in cattle in which only rod-shaped chromosomes were found. It is therefore likely that the interphase chromosomes with the two thick regions correspond to the V-shaped chromosomes of mitosis and that the constriction is actually the spindle attachment.

In a recent paper by Lamb (9) the conclusion is reached that the chromatin threads which we have described "are not isolated chromosomes but are complex fragments of drawn-out nuclei." Lamb's contention will be discussed in another paper.

Composition of Chromosomes.—The three most important constituents quantitatively of chromosomes are desoxyribonucleic acid and two protein fractions, histone and non-histone protein (1). The latter fraction has been called the tryptophane-containing protein because it contains tryptophane and histones do not; and it has been called the residual protein because it is obtained as the residual material after removing histone and DNA. It can be seen from Table I that isolated chromosomes of pancreas, liver, and kidney contain a lower percentage of DNA and a higher percentage of residual protein than do those of the thymus. DNA was determined by the Schmidt-Thannhauser method (10).

TABLE I

Chromosomes of:	Desoxyribonucleic acid	Residual protein
	per cent	*per cent*
Carp erythrocyte	41	4
Calf thymus	39	8.5
Calf liver	26	39
Calf kidney	28	33
Beef pancreas	28	29

Histone.—Histones, being, unlike most other proteins, soluble in sulfuric acid containing mercuric sulfate (0.34 M $HgSO_4$ in 1.88 M H_2SO_4), are released from chromosomes by this reagent and the quantity released may be estimated spectrophotometrically after addition of nitrite (11). In a previous paper it was shown that not all of the histone in the thymus chromosomes is released in this way (1). The quantity that is released is equal to the quantity of DNA present. In all of the chromosomes preparation of which is described in this paper, the quantity of histone released by $HgSO_4$–H_2SO_4 is equal to the quantity of DNA present. In these chromosomes the remainder of the histone has not been estimated.

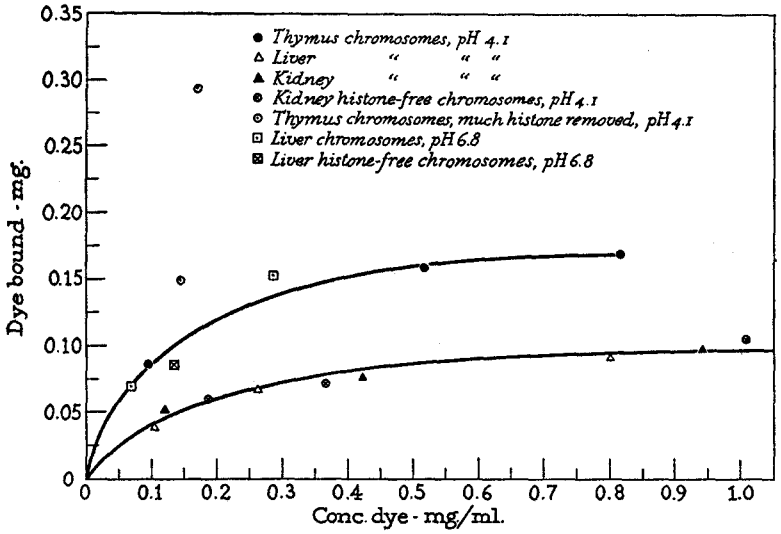
It will be shown in a later section of this paper that all the histone of a chromosome can be removed under certain conditions without affecting its appearance under the microscope. When, on the other hand, either of the other constituents (DNA and non-histone protein) of a chromosome are removed, the microscopic appearance of the chromosome is altogether changed. With these facts in mind, it is of interest to inquire how histone is attached to the chromosome. It is generally supposed that histone, being strongly basic, is combined with DNA; but as Kossel (12) pointed out many years ago, there

actually is no direct evidence for this. "It is still unknown whether a compound of nucleic acid and histone is preformed in the thymus gland" (Kossel, page 73). Evidence for this is provided by a study of the staining of chromosomes with crystal violet.

Crystal violet, being a basic dye, combines with the phosphoric acid groups of DNA. Feulgen (13) found the combination to be stoichiometric; when a solution of DNA was added to a solution of the dye a precipitate was obtained which contained a molecule of dye for every phosphoric acid group. In our experiments the precipitate was washed until practically no dye appeared in the washings, and then the dye combined with DNA was released and measured. The reason for careful washing of the precipitate is that in studying the combination of dye with chromosomes this step is essential. The dye combines loosely with proteins in general but combination is much more selective if after mixing dye and the material to be stained, the latter is destained (a term used by cytologists) with alcohol. Without destaining, crystal violet staining is of little cytological value. In destaining, it is desirable to use a fluid which after a certain point removes negligible quantities of dye so that one knows that destaining is complete. For this purpose we have found isopropyl alcohol a satisfactory reagent. The dye that remains attached after destaining is released by hot ethyl alcohol containing hydrochloric acid and is then measured colorimetrically. It is also important to measure the concentration of dye left in solution when dye is mixed with the material to be stained and after equilibrium has been reached. In referring to the amount of material that is stained, both its dry weight and its DNA content should be known. The importance of the latter is apparent, for proteins do not combine with crystal violet. Of course, if the dye is simply added to a protein, such as coagulated egg albumin, or the residual protein of chromosomes, dye will combine with protein, the amount depending upon the pH of the medium; but if the protein is destained, no dye remains combined. In the present work, by "combined dye" we mean the dye remaining combined after destaining. And by "concentration of dye" we mean the concentration in the suspension of material being stained after equilibrium is reached.

When the combination with crystal violet of free, polymerized DNA is compared with that of isolated thymus chromosomes, it can be seen that in the latter histone prevents most of the DNA from combining with the dye. At a concentration of dye (0.1 mg. per ml.) sufficient to combine fully with free DNA, the DNA in the chromosomes combines with less than one-tenth as much dye. As the concentration of dye is increased, no more combines with the free DNA, but (as shown in Text-fig. 1) more and more combines with the chromosomal DNA until finally (at a concentration of 0.8 mg. per ml.) it has about one-sixth of that held by free DNA. When much of the histone in the chromosomes is removed, the quantity of dye bound increases enormously;

and when extra histone is attached to the chromosomas, almost no dye is bound. For these experiments histone is removed by suspending the chromosomes in 1 M NaCl containing 0.5 per cent of citric acid (pH 2.3). In this way,

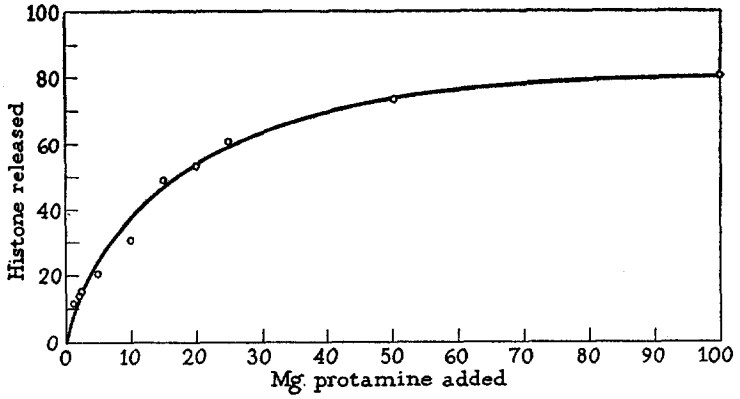


TEXT-FIG. 1. Ordinates on the lower curve represent the amounts of crystal violet bound by liver and kidney chromosomes and by histone-free kidney chromosomes at varying concentrations of dye at pH 4.1 (abscissa). The upper curve represents the amounts of dye bound by thymus chromosomes at pH 4.1. Two of the points (dotted circles) far above the upper curve show how much dye is bound by thymus chromosomes at pH 4.1 when much of their histone has been removed. The points marked with dotted squares indicate the amount of dye bound by liver chromosomes at pH 6.8. Crossed squares show the dye bound by histone-free liver chromosomes. In every experiment the quantity of DNA present is equivalent to a phosphorus content of 0.14 mg. All points on the chart should be compared with the quantity of dye bound by the same amount of isolated DNA. This quantity of dye, too large to be represented on the chart, is 1.05 at both pH 4.1 and 6.8 and does not increase after a concentration of 0.1 mg. dye per ml. has been reached. The difference between the amount of crystal violet bound by isolated DNA and that bound by DNA in a chromosome is an indication of how many phosphoric acid groups of the latter DNA are bound by protein.

much histone but no DNA is extracted, so that the DNA content of the chromosomes is now 60 per cent. The extracted protein was isolated and identified as histone. All the staining experiments that have just been described were done in an acetate buffer at pH 4.1. When they were done in a phosphate buffer at pH 6.8 the results were not as constant but were much the same, showing that at this pH the histone in the thymus chromosomes is combined with DNA.

The staining of chromosomes isolated from other tissues will be described later in this paper.

The curve (Text-fig. 1) showing increased combined dye in thymus chromosomes as concentration of dye is increased suggests that as crystal violet combines with the phosphoric acid groups of DNA it displaces histone. If this happens, as more dye combines histone might appear in solution. In presence of dye, it would, however, be difficult to recognize free histone. The experiment could perhaps be done if instead of a basic dye, a non-pigmented base were used. Experiments were accordingly done using arginine and some aliphatic anines, but no histone was liberated from chromosomes. When salmin, a relatively



TEXT-FIG. 2. Ordinates on the curve represent the quantity of histone released from thymus chromosomes when varying amounts of protamine are added. The histone released is expressed as a percentage of the quantity that is released when the chromosomes are treated with $HgSO_4$–H_2SO_4.

large molecule with many basic groups, was used in experiments like those in which thymus chromosomes are stained with crystal violet, free histone in solution was found. As the concentration of salmin was increased, more and more free histone appeared as it was displaced by protamine from its combination with DNA. The curve relating histone released to protamine concentration is similar to that relating dye combined to dye concentration (Text-fig. 2).

In experiments on the combination of crystal violet with chromosomes a 1 ml. suspension of chromosomes containing 0.14 mg. DNA phosphorus was added to a mixture of 1 ml. buffer, 0.5 ml. 0.14 M NaCl, and 1 ml. dye solution in a 8 ml. test tube. The buffer was either 0.2 M acetate pH 4.1 or 0.2 M phosphate pH 6.8. The crystal violet (National Aniline) was dissolved in water, 4 mg. per ml., and was diluted for most experiments to vary the dye concentration. The chromosomes suspended in the dye mixture were stirred gently at room temperature for 30 minutes. After centrifugation, the supernatant was decanted and the dye concentration in it was determined

spectrophotometrically at 5,900 Å. u. In the meantime the tubes containing the sediment were inverted over filter paper to drain off all the fluid. The sediments were washed with isopropyl alcohol, to every 50 ml. of which had been added 1 drop of acetic acid, the test tube being nearly filled with fluid, stirred for a few minutes, and then centrifuged. Washing was continued until the final supernatant was nearly colorless. Depending upon the concentration of dye used, from 3 to 7 washings were required. After the final washing 5 ml. of acid alcohol (5 ml. HCl for every 200 ml. alcohol) were added to the sediment. The tubes were now placed in a 60° bath for a few minutes and stirred occasionally. After centrifugation the supernatant was a deep blue and the sediment was colorless. The concentration of dye in the supernatant was determined. Every experiment was done in triplicate from the beginning.

In experiments on the combination of DNA with crystal violet a solution of DNA in water containing 0.07 mg. P per ml. was used. The highly polymerized DNA was prepared from the thymus. To 1 ml. of DNA were added 0.5 ml. of pH 4.1 acetate or pH 6.8 phosphate buffer and from 1.0 to 2.0 ml. of a dye solution containing 1 mg. per ml. On addition of dye the DNA precipitated. Dye concentration was determined as in the experiments with chromosomes. After washing the DNA-crystal violet sediment three times, the combined dye was liberated with acid alcohol. The quantity of combined dye increased with the dye added until 1.4 ml. of the latter had been added; a further increase up to 2.0 ml. did not change the amount of dye combined. This was 0.52 mg. and should be multiplied by 2 for comparison with the quantities of dye bound by chromosomes, as shown in Text-fig. 1, for in those experiments twice as much DNA was present.

In experiments on the displacement of histone from chromosomes by salmin, a suspension was used containing 6.5 mg. of thymus chromosomes per ml. of 0.2 M pH 6.8 phosphate buffer. To 1 ml. of this suspension was added 1 ml. of the protamine solution, the latter containing amounts of protamine varying from 1 to 100 mg. The suspension was gently stirred for 2 hours at 25°C. After centrifugation, the histone concentration of the supernatant was determined by adding to it an equal volume of 0.34 M $HgSO_4$ in 1.88 M H_2SO_4, heating at 60° for 15 minutes, adding $^1/_{10}$ volume of 1 per cent $NaNO_2$, heating again at 60° for 15 minutes, cooling, and finally determining the extinction coefficient at 3,540 Å. u. The histone released directly from the chromosome suspension (untreated with protamine) by an equal volume of $HgSO_4$–H_2SO_4 was determined in the same way, and was found to be 40 per cent of the mass of the chromosomes. A solution containing a known amount of histone served as a standard. The quantities of histone displaced from chromosomes by varying concentrations of protamine may be compared with the amount liberated from them by $HgSO_4$–H_2SO_4, as shown in Text-fig. 2.

Residual Protein.—This fraction was prepared from thymus chromosomes by extracting them with neutral 1 M NaCl, which removed all the DNA and histone (1). Residual protein is prepared from kidney, liver, and pancreas chromosomes in two steps; first all the histone is removed with slightly acid (pH 2.9) M NaCl, leaving the DNA and residual protein; then the DNA is removed with a crystalline protease-free preparation of desoxyribonuclease (kindly given to us by Dr. M. Kunitz).

It is important to know whether all the histone is in fact removed by acidified M NaCl. Any histone remaining can be looked for in two places. One place is

in the material removed by desoxyribonuclease. That there is no histone here is shown by adding $HgSO_4$–H_2SO_4, which produces a precipitate, but when nitrite is added no red color appears either in the precipitate or in the supernatant, indicating that no tyrosine-containing protein (and histones contain tyrosine) passes into solution along with depolymerized DNA. The other place to look for histone is in the residue remaining after the action of desoxyribonuclease. To this residue are added $HgSO_4$–H_2SO_4 and nitrite. A brick-red precipitate with a nearly colorless supernatant is obtained, indicating the presence of much protein but no histone, for presence of histone would be revealed by appearance of soluble red pigment.

The insoluble material remaining after treatment with desoxyribonuclease contains about 0.3 per cent P due to a little nucleic acid remaining. The rest of the material is protein, for amino acid analyses (to be reported in another paper (3)) account for nearly all of the nitrogen present. The nitrogen content of different preparations varies from 15.3 to 16.6 per cent. It is this material that we call the residual protein of the chromosome. The residual protein fraction is the total non-histone protein. The quantity of residual protein in a suspension of isolated chromosomes is found by removing histone and then DNA and finally determining the dry weight of the residue, correcting this for the small amount of nucleic acid present.

In Table I are given the residual protein contents of thymus, liver, kidney, and pancreas chromosomes. It can be seen that in relation to DNA content, the amount of residual protein is much greater in liver, kidney, and pancreas chromosomes than in thymus chromosomes. Since it is known that the quantity of DNA per nucleus is constant in the different types of cells (14),[1] it follows that the quantity of residual protein varies considerably in different nuclei, being least in the thymus and considerably more in liver, kidney, and pancreas. In the cells of these tissues the relative amounts of nucleus and cytoplasm also vary greatly, there being far more cytoplasm in cells of liver, kidney, and pancreas than in those of the thymus. Quantitative determinations have recently been made of the relative masses of nuclear and cytoplasmic substance in these tissues (4). It was found that in the thymus the nucleus is 61 per cent of the cell mass, in calf liver 19 per cent, in calf kidney 20 per cent, and in beef pancreas 9 per cent. These figures show that there is a correlation between the quantity of cytoplasm in a cell and the quantity of residual protein in the chromosomes of that cell.

The microscopic appearance of chromosomes has been examined after removal of histone and then again after removal of DNA. Extraction of histone does not affect the appearance of chromosomes. Fig. 3 shows a chromosome from which all histone has been removed. Histone surely does not form the protein

[1] See Addendum at the end of this paper.

thread that, as has long been supposed, is the basis of chromosome structure. Before the presence of other proteins in chromosomes was recognized, the question was raised as to whether protamines and histones are in a fibrous form in chromosomes (15). More recently Mazia (16) and Kaufmann and his coworkers (17) have come to the conclusion that histone is an important part of the fibrous structure of chromosomes. This conclusion, based on the supposition that pepsin does not digest histone in chromosomes, cannot be accepted because it is now known that pepsin does in fact digest histone both when it is isolated and when it is combined in chromatin (18). The fact that all the histones of a chromosome can be removed without affecting its microscopic appearance also shows that its fibrous structure does not depend upon histone.

When chromosomes from which histone has been removed are treated with desoxyribonuclease, DNA and nothing else is removed. This results in a radical change in microscopic appearance of the chromosomes. The tiny coiled threads that remain are difficult to photograph because their thread-like nature is apparent only when one focuses up and down. These minute threads consist of residual protein. If a suspension of chromosomes is treated with an enzyme (crystalline trypsin or chymotrypsin) that digests protein and not DNA, the material becomes gelatinous as DNA is set free from its combination with proteins. Under the microscope scarcely any particulate material can be seen. These experiments show that the microscopic appearance of chromosomes depends on *both* residual protein and polymerized DNA. To form the structure of a chromosome these two constituents must be associated in some way. Since the residual protein is itself a microscopic thread, whereas DNA is not, the protein seems to be the fundamental fiber of chromosomes.

Microscopic study of histone-free chromosomes shows that residual protein and DNA are associated. There is evidence that they are in fact combined. After histone is removed with M NaCl at pH 2.9 the chromosomes are washed with 0.14 M NaCl, to which a little dilute pH 4 acetate buffer is added. The DNA of these chromosomes, when dried, is found to be about 32 per cent for those from liver, and 34 to 39 per cent for those from kidney and pancreas. When a suspension of such chromosomes is allowed to settle practically no DNA is found dissolved in the supernatant, and yet under these conditions DNA itself is quite soluble. It must, therefore, be kept from dissolving by its combination with residual protein. Experiments at pH 4 on the combination of these histone-free chromosomes with crystal violet show that the phosphoric acid groups of DNA are combined with residual protein (Text-fig. 1).

Residual protein and DNA are combined at pH 4 but this does not necessarily mean that they are combined under physiological conditions, for the pH of the nucleus is surely much closer to neutrality. To find whether DNA still remains attached to residual protein in neutral medium, 0.1 M $NaHCO_3$ was slowly added to suspensions of histone-free chromosomes in 0.14 M NaCl

until a pH of 7.5 was reached. Nearly all the DNA remained combined under these conditions, for very little DNA was found dissolved in the supernatant fluid and the DNA content of the chromosomal material dried after washing in saline was only a little less than before neutralization. In two experiments with histone-free pancreas chromosomes, for example, the DNA content at pH 4 of one preparation was 38.4 per cent and of another 34 per cent, while at pH 7.5 they both contained 32 per cent DNA.

Experiments on the combination of histone-free chromosomes with crystal violet show that in neutral media phosphoric acid groups of DNA are combined with residual protein. The quantities of dye bound at pH 6.8 by liver chromosomes before and after removal of histone are shown in Text-fig. 1. It should be said that at pH 6.8 experiments with crystal violet give far less consistent results than they do at pH 4.1. It should also be noted, as previously pointed out in this paper, that under the conditions of these experiments crystal violet does not combine either at pH 4.1 or 6.8 with proteins, not, for example, with residual protein from which DNA has been removed.

A comparison of thymus chromosomes with liver and kidney chromosomes with respect to crystal violet combination shows that removal of histone from the latter chromosomes makes little difference whereas in the former removal of histone greatly increases the quantity of dye bound. This indicates that in thymus chromosomes, where very little residual protein is present, nearly all of the DNA is combined with histone, but that in the other chromosomes residual protein may have a more important part in binding DNA. Even in liver, kidney, and pancreas chromosomes a large part of the phosphoric acid groups of DNA are bound by histone. This can be shown by comparing the appearance of these chromosomes when free of histone at pH 4 and at pH 7. At pH 4 they look just as they do before removal of histone; at pH 7, however, they are swollen and misshapen when examined under the microscope and even a suspension of them in a test tube appears grossly swollen. Just how the phosphoric acid groups of DNA in chromosomes are partitioned between histone and residual protein is not yet known. It is possible that some of the groups of a DNA molecule are combined with histone and other groups of the same molecule are combined with residual protein. In such an arrangement DNA would integrate the chromosomal constituents.

There seem to be two DNA-containing nucleoproteins in chromosomes, for there is evidence that residual protein as well as histone is combined with DNA. After separating these three components there is no difficulty in recombining histone and DNA, but in neutral medium residual protein combines with no more than traces of DNA. Failure of isolated residual protein to combine with DNA is probably due to the fact that once they have been separated, the protein threads coil up and also aggregate together, thus covering groups that had been combined with DNA. That coiling and aggregation may cover up

groups of proteins and so render them unreactive has been recognized before (19, 20). Another possible explanation of why isolated DNA and residual protein do not combine when mixed together is that DNA and residual protein in the intact chromosome are not combined and that their combination in histone-free chromosomes is altogether an artifact.

The procedure for preparing histone-free chromosomes and residual proteins will now be described. In the example given kidney chromosomes were used, but for liver or pancreas chromosomes the procedure would be the same. In this experiment 92 ml. of a chromosome suspension in 0.2 per cent citric acid was used. This suspension contained 727 mg. of chromosomes, the DNA content of which was 27.4 per cent. To it were added 92 ml. of 2 M NaCl and then 184 ml. of 1 M NaCl. The suspension was stirred gently for several hours and then centrifuged for 30 minutes at 3,500 R.P.M. in a head 40 cm. in diameter. A clear supernatant was obtained. The ultraviolet absorption of the supernatant showed that it contained protein with a trace of nucleic acid. The sediment was washed with M NaCl. The chromosomes were examined microscopically while in M NaCl and were found to be well formed. They were then washed in 0.14 M NaCl and brought to a volume of 69.5 ml., each milliliter of which contained 5.8 mg. of dry salt-free material, which is equivalent to 54.5 per cent of the weight of the original chromosomes. The dry weight was obtained by washing with 66 per cent alcohol to remove salt and then dehydrating with hot alcohol and ether and drying at 106°C. The DNA content of these histone-free chromosomes was 38.7 per cent.

To 64.5 ml. of the suspension of histone-free chromosomes in 0.14 M NaCl were added more 0.14 M NaCl and about 7 ml. of 0.1 M $NaHCO_3$ to bring the pH to 7.0. The suspension, volume about 140 ml., was stirred for 10 minutes and centrifuged for 15 minutes at 7,000 R.P.M. in a head 26 cm. in diameter. The supernatant was clear. The phosphorus contant of the supernatant showed that it contained about 4.5 per cent of the DNA in the chromosomes. To the sediment were added 0.14 M NaCl containing enough dilute NaOH to make the pH 7.4, 0.5 ml. 1 M $MgCl_2$, and 5 ml. 0.14 M NaCl containing 2 mg. of crystalline desoxyribonuclease—total volume 64.5 ml. This suspension, to which a few drops of toluene were added, was kept at 30°C. for 2 hours with occasional mixing. It was then centrifuged. The phosphorus content of the supernatant accounted for 95 per cent of the total phosphorus present. A sample of the supernatant was then treated with $HgSO_4$–H_2SO_4 and nitrite. A white precipitate and a colorless superantant were obtained, showing that no histone or other protein was present. The residue was washed with 0.14 M NaCl and brought to a volume of 20 ml. The dry salt-free weight was 11.4 mg. per ml. This material contained 2.8 per cent of nucleic acid. The remainder was protein, the residual protein. This protein was equivalent to 60.3 per cent of the weight of the histone-free chromosomes and accounted for all the protein in these chromosomes, for their DNA content was 40 per cent. The residual protein was 33 per cent of the weight of the original chromosomes.

SUMMARY

1. The preparation of isolated chromosomes from liver, kidney, and pancreas has been described.

2. It has been shown that there is no gross cytoplasmic contamination in these preparations.

3. In a microscopic study of isolated chromosomes the same chromosomes have been found in different tissues of the same organism. Since individuality is one of the main characteristics of chromosomes, there can be little doubt that the preparations do, in fact, contain isolated chromosomes.

4. A quantitative study of staining with crystal violet shows that this basic dye competes with histone for the phosphoric acid groups of the DNA in chromosomes. The displacement of histone by protamine has been demonstrated.

5. Preparation of histone-free chromosomes has been described. Removal of histone does not affect the microscopic appearance of chromosomes.

6. The non-histone or residual protein has been prepared from histone-free chromosomes. The quantity of residual protein in a preparation of chromosomes is correlated with the amount of cytoplasm in the cells from which the chromosomes were prepared.

7. The microscopic appearance of chromosomes depends upon the association of DNA with residual protein.

8. Evidence has been given that in a chromosome there are two DNA-containing nucleoproteins; in one DNA is combined with histone, and in the other it is combined with residual protein.

Addendum.—The observation that the quantity of DNA in different somatic nuclei of the same organism is constant and twice that in sperm cells was made independently by Boivin, Vendrely, and Vendrely (21) and by Mirsky and Ris (14), although publication by the former authors came first. The genetic significance of DNA constancy was recognized by both groups of authors. Mirsky and Ris showed that constancy held for the cell of seven different vertebrate species, but their analyses for the cells of beef tissues led them to suppose that these cells form an exception to the rule of constancy.

A further step was the demonstration by Ris and Mirsky (22) that the Feulgen reaction can be used quantitatively for DNA determinations on single nuclei in microscopic preparations. In this *cytochemical* procedure no attempt was made to determine the absolute quantity of DNA in a nucleus; relative DNA contents, however, were determined for cells of different species and for different cells of the same species. The validity of such cytochemical determinations was established by what may be called a *chemical* procedure, in which the amount of DNA in a counted suspension of isolated nuclei was determined and this amount divided by the number of nuclei to give the quantity of DNA per nucleus. That the cytochemical procedure gives correct relative determinations was shown by the fact that the same relative values for a series of nuclei were obtained by the chemical procedure. Using the cytochemical method, Ris and Mirsky showed that when no polyploidy occurs different somatic nuclei of the same organism have constant amounts of DNA, but that in mammalian liver, in which polyploidy occurs, the quantity of DNA per nucleus is proportional to the number of sets of chromosomes present.

Subsequent to the work of Ris and Mirsky there appeared a paper by Swift (23) in which a similar cytochemical procedure (though with some useful modifications), was described and many similar cytochemical determinations were mentioned. A series of determinations made by Swift, and not by Ris and Mirsky, showed that, apart from polyploidy, different somatic cells of the mouse have the same quantity of DNA and he noted the same constancy for the DNA of beef tissue cells. This showed that Mirsky and Ris were probably in error when they supposed that beef cells were an exception to the many examples of constancy which they had found.

In a recent paper by Leuchtenberger, Vendrely, and Vendrely (24) cytochemical and chemical determinations are compared in essentially the same way for various nuclei of beef as Ris and Mirsky had previously done for nuclei of other animals. Leuchtenberger *et al.*, in confirmation of Swift, find that constancy holds for beef tissues. In referring to the original paper by Mirsky and Ris on constancy of DNA emphasis is misplaced in such a way that the false impression easily could be had that Mirsky and Ris were not codiscoverers of the constancy of DNA but, on the contrary, among the main opponents of this idea. A curious omission by Leuchtenberger *et al.*, is that no mention is made of the paper by Schrader and Leuchtenberger (25) in which they attacked the idea of DNA constancy.

A. E. MIRSKY

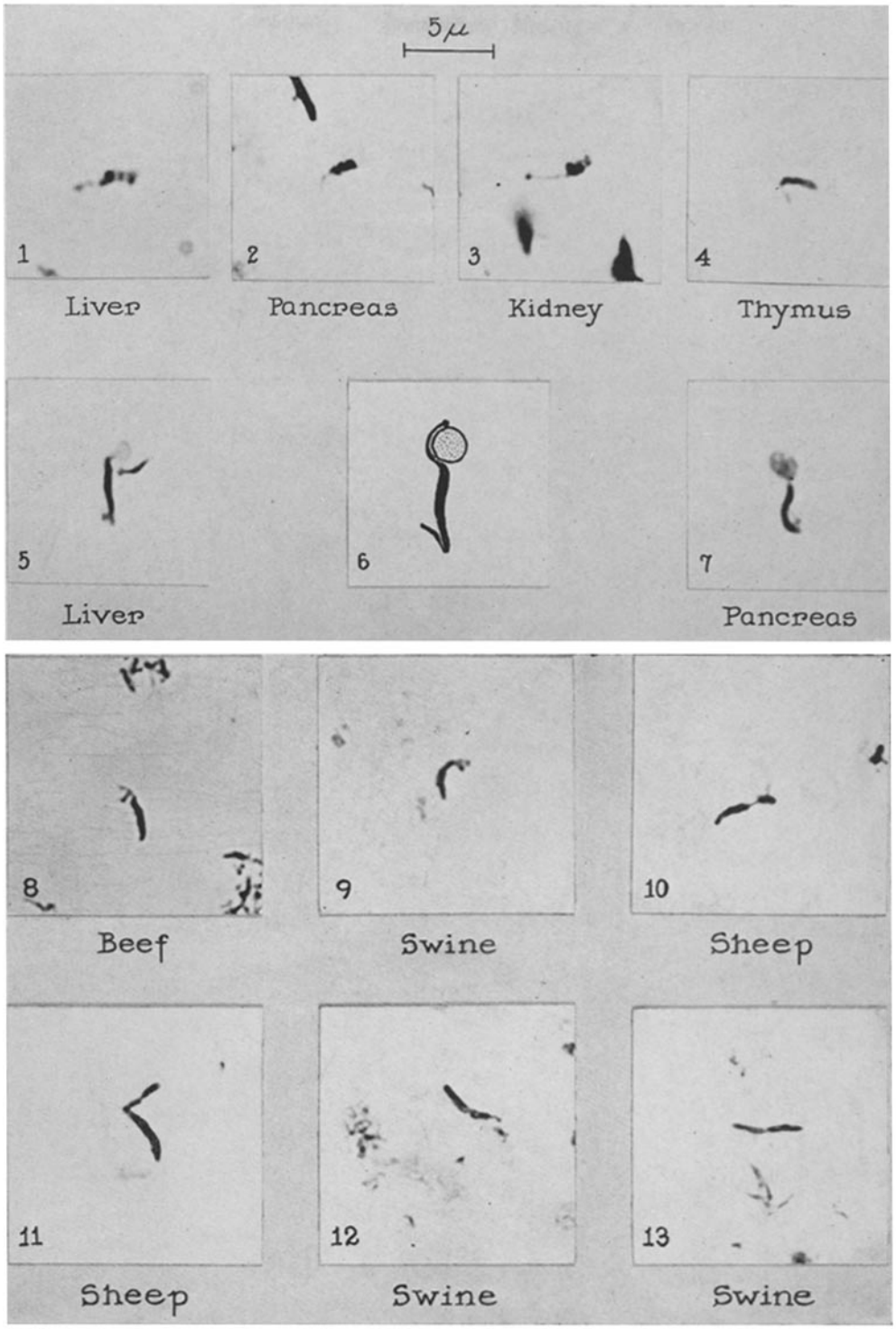
REFERENCES

1. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1947, **31,** 1.
2. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1949, **178,** 53.
3. Daly, M. M., and Mirsky, A. E., unpublished experiments.
4. Allfrey, V. G., Mirsky, A. E., and Stern, H., unpublished experiments.
5. Heitz, E., *Planta*, 1931, **12,** 774.
6. McClintock, B., *Z. Zell forsch. u. mikr. Anat.*, 1934, **21,** 294.
7. Boveri, T., Ergebnisse der chromatischen Substanz des Zellkerns, Jena, Gustav Fischer, 1904.
8. Makino, S., *Cytologia*, 1944, **13,** 247.
9. Lamb, W. G. P., *Nature*, 1949, **164,** 109.
10. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1945, **161,** 83.
11. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, 1946, **30,** 117.
12. Kossel, A., The Protamines and Histones, New York, Longmans Green and Co. 1928.
13. Feulgen, R., *Z. physiol. Chem.*, 1913, **84,** 309.
14. Mirsky, A. E., and Ris, H., *Nature*, 1949, **163,** 666.
15. Waddington, C. H., An Introduction to Modern Genetics, New York, Macmillan Co., 1939.
16. Mazia, D., *Ann. New York Acad. Sc.*, 1950, **50,** 954.
17. Kaufmann, B. P., Gay, H., and McDonald, M. R., *Cold Spring Harbor Symp. Quant. Biol.*, 1949, **14,** 85.
18. Daly, M. M., Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1951, **34,** 439.
19. Mirsky, A. E., and Pauling, L., *Proc. Nat. Acad. Sc.*, 1936, **22,** 439.
20. Mirsky, A. E., *Cold Spring Harbor Symp. Quant. Biol.*, 1941, **9,** 278.

21. Boivin, A., Vendrely, R., and Vendrely, C., *Compt. rend. Acad. Sc.*, 1948, **226,** 106.
22. Ris, H., and Mirsky, A. E., *J. Gen. Physiol.*, 1949, **33,** 125.
23. Swift, H. H., *Physiol. Zool.*, 1950, **23,** 169.
24. Leuchtenberger, C., Vendrely, R., and Vendrely, C., *Proc. Nat. Acad. Sc.*, 1951, **37,** 33.
25. Schrader, F., and Leuchtenberger, C., *Proc. Nat. Acad. Sc.*, 1949, **35,** 464.

EXPLANATION OF PLATE 2

FIGS. 1 to 13. The isolated chromosomes shown were all fixed and stained in aceto-orcein. They were photographed with a 2 mm. Zeiss apochromat, 15 × ocular—magnification 1200. Since they were enlarged 2 × photographically, the final magnification is 2400. Fig. 6 is a schematic drawing of the nucleolar chromosomes.



(Mirsky and Ris: Composition and structure of isolated chromosomes)