PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Basic Proteins of Cell Nuclei

E. Stedman and Ellen Stedman

Phil. Trans. R. Soc. Lond. B 1951 **235**, 565-595
doi: 10.1098/rstb.1951.0008

THE BASIC PROTEINS OF CELL NUCLEI

By E. STEDMAN, F.R.S. and ELLEN STEDMAN
Department of Biochemistry, University of Edinburgh

(*Received* 15 *May* 1951)

[Plate 20]

Cell nuclei have been isolated from various types of tissues. Evidence is presented which indicates that no appreciable loss of their major components occurs during isolation. Nuclei from normal cells of a particular type possess a constant composition characteristic of that type.

The basic proteins extracted from such nuclei are not homogeneous. When they are of the histone type they can be separated into two parts conveniently described as a main histone and a subsidiary histone fraction respectively. A subsidiary product has also been obtained from protamins.

A study of the chemical composition of the main histones and the protamins has shown that some of these are unquestionably species-specific. The phenomenon of cell specificity of these basic proteins has also been established in a number of instances.

One case of cell specificity has also been found among the subsidiary histones. The difficulty of purification has prevented the detailed examination of others.

The possible physiological significance of cell specificity of the basic proteins of cell nuclei is discussed.

Introduction

During the past decade the present authors have been engaged on an investigation of the chemical nature of the cell nucleus, the general results of which have, at the invitation of various institutions, been recorded elsewhere (Stedman 1944; Stedman & Stedman 1947*a*, *b*). Concurrently with this general survey of the problem, a more detailed examination of the individual components has been in progress. That part dealing with the basic proteins, while it has by no means reached finality, has nevertheless now furnished results of sufficient interest to warrant description. The main object of the present communication is to record these results and to discuss their implications. The work, however, also constitutes a contribution to our general study of the chemical components of cell nuclei, for all the compounds examined have been prepared from isolated cell nuclei and are hence components of these cell organs. Advantage has therefore been taken of the opportunity thus presented of describing the methods used for the preparation of the cell nuclei from which the basic proteins have been obtained, and to present evidence indicating the extent to which they correspond in composition with the intact nucleus in the cell.

It has long been known as a result of the classical researches of Miescher (1897) and Kossel (1884) that two types of basic proteins, protamins and histones, are components of the nuclei of certain animal cells. In his last review of the subject, Kossel (1928) expressed the opinion that these basic proteins do not occur in all cell nuclei, 'but only in the nuclei of certain kinds of tissue'. What limitations in their distribution Kossel had in mind is not disclosed, but it must be presumed from other of his statements that he

believed that they were present only in fish sperm heads and in the nuclei of avian erythrocytes and of thymocytes, for it was, in fact, only in these nuclei, or in the tissues containing them, that they had been found. It has now become recognized as a result of the present authors' work in Britain and of that of Mirsky and his collaborators in America that these basic proteins are much more widespread in their occurrence. They have been extracted as sulphates from nuclei (Stedman 1944) isolated from various tissues, including tumours, and in the form of nucleoproteins (Mirsky & Pollister 1942, 1943), or as components of thread-like structures which have been termed 'chromosomes' (Mirsky & Ris 1948), obtained directly from a number of tissues. While this work has not embraced every known type of cell, there can be little doubt that basic proteins are components of all cell nuclei in the higher animals.

During the course of this work, it was observed (Stedman & Stedman 1944) that the nuclei of the liver cells and erythrocytes of the salmon differed from the sperm heads of this species in containing histones. This was contrary to expectations, for, while protamins were known to occur in the sperm heads of a number of species of fish, others, in particular the cod, contain a histone. This, coupled with the fact that the two types of basic protein have never been observed to occur together in the same cell, suggested that their biological functions are identical and that consequently the chemical differences which distinguish salmine (salmon protamin) from cod histone are merely expressions in an unusually marked form of the species differences which are known to occur between homologous proteins of different species. Since, moreover, there was no reason for suspecting cell specificity in the basic proteins of a given species, it was anticipated that the nuclei of the somatic cells of the salmon would all contain a protamin identical with that in the sperm heads. Not only did this anticipation prove to be incorrect, but there appeared to be a difference, quite outside the limits of experimental error, between the histones isolated from the nuclei of the erythrocytes and the liver cells. The presence of a histone in the nuclei of the liver cells of the salmon has been confirmed in the present investigation, but owing to the difficulties experienced in procuring specimens of salmon blood it has not been possible to reinvestigate the composition of the histone from the erythrocytes. The problem has therefore been examined in other species, with the results recorded in this communication. A brief, preliminary account of some of the results has been published elsewhere (Stedman & Stedman 1950*a*).

Isolation of cell nuclei

In order to ensure that the basic proteins used in this work were actual components of the nucleus, they were, with one exception, extracted from isolated cell nuclei. No single, general method for the isolation of cell nuclei, universally applicable to all types of cells, is available. Three different procedures have therefore been used respectively for fish spermatozoa, nucleated erythrocytes and cells present in compact tissues such as thymus glands, livers and tumours.

Fish sperm heads

Fish sperm heads, which appear microscopically to be naked nuclei, can sometimes be isolated by a technique which, in its main points, is identical with that originally employed

by Miescher (1897). Seminal fluid, obtained by stripping the live fish, is no doubt the most favourable material to employ, but this is unfortunately difficult to procure. The mature testes must, therefore, usually suffice. If these are of a large size, as with the salmon, the semi-fluid contents can be readily expressed from them. The material so obtained is then suspended in five times its volume of water and the suspension stirred vigorously with a mechanical stirrer for about 15 min., after which it is strained through fine muslin. It is then made just acid to litmus by the addition of dilute acetic acid and, after about 15 min., centrifuged at 3000 r.p.m. for 15 to 20 min. The sperm heads, admixed with a small amount of fibrous impurity, usually sediment readily. If they fail to do so, more acetic acid must be added. The supernatant fluid, which at this stage is turbid, is rejected and the sediment purified by suspending it in water, preferably acidified with dilute acetic acid to assist sedimentation, and again centrifuged. This process is then repeated until the supernatant fluid is perfectly clear and the sperm heads, when examined microscopically, appear to be homogeneous. It has been our practice to effect the rapid microscopic examination of the material during the preparation by the use of wet smears. A drop of the suspension is mixed on a slide with a drop of a solution of methylene blue, covered with a cover-slip, and immediately examined in the microscope. An illustration of such a wet smear of a finished preparation of salmon sperm heads is shown in figure 1, plate 20. Figure 2, plate 20, shows another preparation, more highly magnified, after treatment with 70 % alcohol. If microscopic examination reveals the presence of impurities when no turbidity is visible in the supernatant fluid, the speed and time of centrifugation must be diminished in subsequent fractionations. This may, however, result in the failure of a small proportion of the sperm heads to sediment. When the fractionation is complete, the preparation is dried by suspending it with intimate mixing in about 5 vol. of alcohol. After standing overnight, this treatment is repeated with fresh alcohol. The solid is then finally suspended in dry ether and filtered on a Buchner funnel with suction.

When this method is applied to the testes of the herring, of which, owing to their small size, many are required, it is inconvenient to express the seminal fluid from them individually. The testes are therefore minced coarsely in a machine and the resulting pulp suspended in water and stirred as before. Fragments of the membranes are then removed by straining the mixture through a fine wire gauze, after which the procedure follows the above description. The same mincing process must also be applied to testes from the salmon which are not quite mature in the sense that their contents are not fluid. Contrary to the general belief, such testes obtained at the approach of the spawning season yield good specimens of sperm heads, although the fractionation process is prolonged, and the purification rendered more difficult, owing to the greater bulk of fibrous impurities which must be removed.

The preparation of sperm heads from the testes of the cod presents more difficulty. This is doubtless due to the more complicated structure of the testes, as compared with those of the salmon and herring, and possibly also because the maturation does not proceed uniformly throughout the numerous lobes. The minute size of the sperm heads, which renders their purification by fractional centrifugation difficult, is also a contributory factor. In the preparation of sperm heads from this material, the lobes of the testes

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

are removed from the remainder of the tissue, rejecting those which are small and obviously immature, and minced. Purification is then effected by the method described above. It is, however, usually impossible to remove the impurities completely by fractional centrifugation. Minute fibres, tangled together and enmeshing some of the sperm heads, sediment in the centrifuge at least as readily as the free heads. The fibres are therefore liberated, and the clumps of nuclei which they cause broken up, by suspending the material in four volumes of 4 % acetic acid and stirring the suspension vigorously with a mechanical stirrer. The fluid is then again strained through fine muslin and the sperm heads sedimented in the centrifuge. The subsequent process of fractional centrifugation, which is continued until the fibre is completely removed, is effected with 1 % acetic acid, each fresh suspension in this liquid being stirred before centrifugation so long as clumps of mixed sperm heads and fibre are present. When the preparation is obtained free from fibre it is dried with alcohol and ether by the usual procedure.

Nucleated erythrocytes

All methods for the preparation of nuclei from nucleated erythrocytes depend upon laking the washed cells and removing the haemoglobin by washing the nuclei repeatedly with physiological saline. Different laking agents have been used. Ackermann (1904), for example, used water. The common procedure, however, is to employ saponin. We have used both these methods and have also occasionally substituted cetavlon for saponin. For various reasons none of the methods has proved to be uniformly successful, many of the difficulties experienced being due to the great resistance of a small proportion of the cells to the laking agent. In all our experiments sodium oxalate (0·2 g./100 ml. blood) has been used as anti-coagulant. The oxalated blood was centrifuged and the corpuscles washed three times with physiological saline in a volume approximately equal to that of the plasma removed. When water was used as a laking agent, a measured volume was added to about half its volume of corpuscles and the mixture stirred with a glass rod until it became dark in colour and clear to the unaided eye, a change which occurred in about 1 min. A volume of a 1·7 % solution of sodium chloride equal to that of the water used was then added and the mixture again stirred. The mixture was then centrifuged, the dark red supernatant solution rejected and the residue washed with physiological saline until it was evident from the presence of red corpuscles beneath the mass of yellow nuclei that laking had been incomplete. Even after repeating this treatment several times some erythrocytes remained unlaked. Meanwhile, however, changes in the nuclei had occurred. In one experiment with fowls' blood, a proportion of the nuclei had coalesced together in the form of jelly-like masses. In this particular case it was possible to remove these by straining a suspension of the nuclei in saline through muslin. The suspension was then centrifuged at low speed for about a minute, which caused the remaining unlaked corpuscles to collect at the bottom of the tube leaving the nuclei in a sufficiently loose state in the supernatant fluid to be pipetted from the corpuscles. This experiment yielded a serviceable preparation of nuclei, but in all other and subsequent applications of this technique to fowls' blood, the nuclear material coalesced completely to a viscous mass which appeared to contain no whole nuclei whatsoever. It was, moreover, contaminated with haemoglobin, which could not be removed by washing with

saline. Similar results were obtained in two experiments with blood from the salmon, but with this material laking was fortunately complete, and it proved possible to remove the haemoglobin completely from the product by washing with saline. This product served as the nuclear material from which histone was isolated in our earlier experiments.

In most of our preparations of nuclei from erythrocytes haemolysis has been effected by saponin. In a few cases, however, cetavlon has been used. An identical procedure has been adopted with both haemolytic agents. The washed corpuscles from fowls' blood are mixed with 2 vol. of a 0·3 % solution of the haemolytic agent in physiological saline and stirred for about 2 min. An equal volume of saline is then added and the mixture allowed to stand for 15 min. It is then centrifuged and the residue washed several times with physiological saline. A small proportion of intact erythrocytes is always present at this stage. These residual cells cannot be haemolysed by short contact with the haemolytic agent. It is necessary to leave them overnight (about 12 hr.) with a 0·3 % solution of agent, when haemolysis is complete and the haemoglobin can be completely removed by washing the nuclei with physiological saline. Even with this method, however, some jelly-like particles, presumably formed by the coalescence of some of the nuclei, are present. These can be removed by straining the suspension through muslin. The nuclei are then washed successively with 50 %, 70 % and absolute alcohol, the washing with the latter solvent being repeated as many times as is necessary completely to remove the yellow pigment which is usually, but not invariably, associated with fowl erythrocyte nuclei. They are then suspended in ether and filtered at the pump. Microscopic examination of nuclei prepared by this method reveals the fact that a proportion of the nuclei, although free from haemoglobin, retain the stroma. Thus, in wet smears of the undried preparations an outline ('ghost') of the whole cell, retaining its original oval shape and containing a nucleus in the centre, can sometimes be seen, while in preparations treated with alcohol or completely dried a fine jagged protruberance, evidently consisting of part or all of the stroma, juts out from the surface of many, but not all, nuclei. The proportion of nuclei retaining stroma can, we believe, be considerably diminished by vigorously stirring a suspension of the nuclei from the final saline washing in 4 % acetic acid, straining through muslin and repeating the treatment on the centrifuged material with 1 % acetic acid. We have, in fact, adopted this procedure in most of our preparations. One disadvantage of the use of haemolytic agents lies in the apparent ability of the nuclei to retain a small quantity of them. That saponin is so retained has been shown in some unpublished determinations of furfurol-yielding substances which Dr A. B. Roy has carried out in his laboratory on some of the above preparations. The results show that nuclei from fowl erythrocytes prepared with the aid of saponin yield considerably more furfurol than do those obtained by other means. It is clear that this extra furfurol originates from saponin retained by the nuclei.

The saponin method has only been used on one specimen of erythrocytes from the salmon, and this was unfortunately too small to be of further use in the present investigation. It is mentioned here, however, because of its anomalous behaviour. On centrifuging the liquid after treatment of the washed cells with saponin two layers were obtained: a lower one comprising the bulk of the material and consisting of a red gel, and a small upper one of colourless liquid. It appeared as if the gel had been formed

by the combination of the haemoglobin with the nuclear material under the influence of the saponin. On breaking up the gel with a rod, stirring it with saline and again centrifuging, the volume of the gel diminished and the saline removed some of the haemoglobin. By repeating this process several times the nuclear material was obtained as a colourless jelly which yielded a white solid on drying.

Compact tissues

Nuclei from compact tissues are obtained by treating the pulp with 4 % acetic acid, straining the product and fractionally centrifuging it in 1 % acetic acid according to the procedure recently described elsewhere (Stedman & Stedman 1950*b*). In applying this method to the thymus glands and spleens of the fowl, it was necessary, owing to the small size of these organs, to use material from not less than three dozen chickens in each preparation. In order to avoid deterioration during the collection of this material, each gland was frozen hard in solid carbon dioxide immediately after being dissected out of the animal and maintained in this condition until used. Storage in this condition was never prolonged beyond 3 days. The glands from about 150 chickens were eventually required to provide sufficient material for this investigation. Salmon livers, sent to us from a distance, were similarly stored in solid carbon dioxide during transit.

Preparation of nuclear material from wheat germ

Owing to the presence of the cellulose cell membrane, it has not proved possible to isolate nuclei from plant material by methods similar to those used with animal tissue. In order to confirm the presence of basic proteins in plant cell nuclei and to isolate such a protein for examination, nuclear material has been prepared from wheat germ by a method based on that of Mirsky & Pollister (1942).

Some non-nuclear material is first removed by suspending the wheat-germ (600 g.), previously ground in a mortar, in 2 l. of physiological saline and stirring the mixture for about 15 min. The material is then centrifuged for 20 min. at 2000 r.p.m. The turbid supernatant liquid is discarded and the extraction process repeated on the solid. After this second extraction, the solid is stirred with 1 l. of 10 % sodium chloride, allowed to stand with occasional stirring for 6 hr. and centrifuged at 2500 r.p.m. for 20 min. The viscous, yellow supernatant liquid is strained through muslin to remove the coarser of the particles which are still suspended in it. A second extract is prepared from the solid residue in the same way and the two combined extracts are clarified in a Sharples' centrifuge. This removes some small yellow particles, presumably of cytoplasmic origin, together with a finely divided, buff-coloured substance. The nucleoprotein is precipitated from the clarified extract by slowly pouring into it with continuous stirring 3 vol. of water. The viscous nucleoprotein separates and rises to the surface as a coherent mass. It can be readily removed from the slightly turbid liquid by collecting it on muslin. By way of purification, it is redissolved in 10 % sodium chloride and the solution, after clarification, reprecipitated, a process which can be repeated two or three times. But with each successive precipitation the nucleoprotein becomes less coherent, and hence more difficult to collect, and finally fails to precipitate at all. It is evident from this behaviour that each precipitation is associated with a loss of protein.

Effect of isolation procedure on the chemical composition of cell nuclei

It has been tacitly assumed in our former investigations (Stedman & Stedman 1947*b*) that the cell nuclei isolated by the acetic acid procedure mentioned above retain intact their major components. Any lipids which may have been present are deliberately removed by the alcohol-ether treatment, but, apart from compounds of this type, it has been assumed that little is lost from the nucleus except, perhaps, inorganic material and, possibly, small amounts of substances of low molecular weight which do not combine with nucleic acid. There were two reasons for this assumption; the isolated nuclei, when examined microscopically, appear to be identical with the corresponding nuclei in histological sections; they also react to staining procedures in much the same way. Thus, as is evident from an illustration of one of our preparations of ox liver nuclei shown elsewhere (Stedman & Stedman 1950*b*), the nucleoli and 'chromatin' particles were still present and retained their capacity to take up basic dyes. Secondly, analyses of numerous preparations of nuclei from a particular organ of one species gave, within narrow limits, identical results, which were, moreover, consistently different from the similarly identical results obtained from another organ. Not only was this the case, but morphologically identical nuclei from several species of mammals were found to contain corresponding components in almost the same proportions. Thus, the nuclei of lymphocytes obtained from the spleens of mice contained virtually the same percentage of nucleic acid and histone as did nuclei from the thymocytes of the calf and man. The same correspondence obtained between the nuclei of liver cells from the ox and the mouse. Taken altogether, these results constitute prima facie evidence of the validity of our assumption. Pollister & Leuchtenberger (1949) have, however, pointed out that whereas our isolated nuclei contain protein and nucleic acid in a ratio of about 2, the values determined by cytological absorption measurements on various interphase and prophase nuclei range from 6·5 to 20. If we take a ratio of 10:1 as the most common value obtained by the cytological procedure, this would mean that, during the period of isolation, our nuclei had lost 80% of their content of protein or about 75% of their dry weight. That nuclei could retain their characteristic structure after sustaining such a loss in their substance is, we believe, manifestly impossible. But since, according to Pollister & Leuchtenberger, 'there seems [from their experiments] good reason to suppose that the more drastic procedures [for isolating cell nuclei] would reduce the protein content of nuclei' to the values of those of our preparations, we describe below experiments, carried out some years ago during the period when we were exploring methods for the isolation of cell nuclei, which show that our procedures do not, as the above authors suggest, produce any significant change in the composition of cell nuclei.

The first of these experiments concerns fowl erythrocyte nuclei. As pointed out above, our practice is to lake the erythroyctes with a solution of a haemolytic agent in saline. This does not, as does the method used with compact tissues, necessitate the treatment of the nuclei with 4% acetic acid. It nevertheless seemed desirable in order to facilitate their subsequent chemical examination to submit them to this treatment. One batch of nuclei obtained by laking the corpuscles with cetavlon and removing the haemoglobin with saline was divided into two portions, one of which was dried with alcohol and ether

while the other was stirred with 4 % acetic acid for 15 min. and left in this solvent overnight before being so dried. Unfortunately, the former (saline) preparation was dried directly with absolute alcohol without prior washing with aqueous alcohol and was consequently contaminated with sodium chloride. The nucleic acid contents of, and yields of histone chloride from, the two preparations were: saline, 34·5 % nucleic acid, 25·3 % histone chloride; acid, 38·1 % nucleic acid, 28·0 % histone chloride. The small differences between the results can clearly be accounted for by the contamination of the saline preparation with sodium chloride. This is confirmed by the value of 37·1 % obtained for the nucleic acid content of each of two other saline preparations isolated under the same conditions except for the use of saponin as haemolytic agent and aqueous alcohol for removing the sodium chloride. It is clear that treatment of nuclei with 4 % acetic acid during the isolation process does not materially affect their composition.

A second experiment was made with ox liver. The original purpose of this experiment was to ascertain if the purity of nuclei prepared from compact tissues could be improved by washing the tissue pulp with saline prior to its treatment with 4 % acetic acid. Our final preparations of dry nuclei are, in general, almost white, but those obtained from liver usually exhibit a very faint brown tinge, probably due to the adsorption of small amounts of degradation products of haemoglobin. By washing the minced tissue with saline it was hoped to avoid this contamination. The finely minced liver (about 4 lb. in weight) was divided into three portions 1, 2 and 3. Of these 1 and 3 were stirred vigorously for 15 min. with a mechanical stirrer (not blender) with about two volumes of 0·85 % sodium chloride. The mixture was then centrifuged at 2000 r.p.m. for 20 min., the coloured supernatant fluid, which contained many small granules in suspension, rejected and the sediment again stirred, this time manually, in the centrifuge tubes with more saline. After centrifuging, washing in the latter manner was twice repeated on the residue, the material thus having been subjected to a total of four extractions with saline. Portion 1 was then treated with 4 % acetic acid and worked up by the customary procedure. Owing to the strain on our resources of apparatus caused by the attempt to prepare three separate batches of nuclei simultaneously, it was necessary to leave portion 3 in saline for about 6 hr.; otherwise, the treatment it was subjected to was identical with that described for portion 1. Portion 2 served as a control. It was worked up without delay by our normal acetic acid procedure, i.e. without the preliminary washings with saline to which 1 and 2 were subjected. The finished preparations of nuclei differed little in microscopic appearance. The proportion of contaminating cytoplasmic granules was, if anything, slightly smaller in the preparations washed with saline, but it was, in any case, negligible in amount in all preparations. The dried products did, however, exhibit some difference in colour, the saline preparations being of a purer white than the other. Analyses gave the following results for the nucleic acid contents and yields of histone sulphate: no. 1, 30·7 % nucleic acid, 25·5 % histone sulphate; no. 2, 28·7 % nucleic acid, 24·0 % histone sulphate; no. 3, 30·5 % nucleic acid, 27·0 % histone sulphate. It is clear that the two saline preparations are, within the limits of experimental error, identical in composition, despite the widely different lengths of time with which the tissue from which they were prepared were in contact with the 0·85 % solution of sodium chloride. The normal preparation, however, contained slightly smaller amounts of both nucleic

acid and histone: these differences were certainly outside the limits of experimental error with the former and probably with the latter component. Nevertheless, the differences are small. In our opinion they are best interpreted as due to the greater freedom from contamination of the nuclei prepared with the use of saline. We have not, however, incorporated this saline procedure into our routine method for preparing cell nuclei from compact tissues because the slight increase in purity which results does not seem to justify the much greater labour which it involves.

Another experiment bearing on this theme is one which was designed to test the efficacies of different concentrations of acetic acid in cytolyzing cells. The pulp obtained by finely mincing about 2 lb. of ox liver was divided into two portions, which were treated with 4 and 1 % acetic acid respectively. On microscopic examination of the products during the process of fractional centrifugation it was observed that, while the 4 % acetic acid had effected a virtually complete liberation of the nuclei, the 1 % acid had been so ineffective in this respect that the nuclear fraction contained a large proportion of whole liver cells. Nevertheless, both preparations were brought as far as possible to completion, The 4 % acid treatment gave a normal good preparation of nuclei free from serious contamination in a yield of 2·1 g. (air-dry weight). On the other hand, the 1 % acid treatment gave a product contaminated with a small proportion of whole liver cells in a yield of 1·75 g. Analyses gave the following results: 4 % acid, 28·7 % nucleic acid. 22·5 % histone sulphate; 1 % acid, 27·0 % nucleic acid, 21·5 % histone sulphate. The difference between the yields of histone is not significant, but the smaller content of nucleic acid in the 1 % acetic acid preparation is obviously attributable to the presence in it of the visible cytoplasmic contamination. Besides demonstrating the superiority of the 4 % acid as a cytolyzing agent, the experiment also shows that it produces no more change in the composition of the nuclei than does the 1 % acid.

It is a fortunate circumstance that, while the nuclei of avian erythrocytes cannot be isolated without subjecting them to the action of saline, the nuclei from liver cells do not require this treatment but must, in the procedure which we employ, be treated with 4 % acetic acid. For this difference between the treatments required by the two types of tissue has enabled us to test in the above experiments the solvent action of 4 % acetic acid on nuclei which have been isolated in a medium of saline, and that of saline on finely minced tissue from which the nuclei can subsequently be liberated by treatment with 4 % acetic acid. It is evident from the results that, if one rejects as wholly improbable the possibility that the two liquids exert precisely identical solvent effects, neither 0·85 % sodium chloride nor 4 % acetic acid removes any appreciable amount of the major components from cell nuclei. Taken in conjunction with the other evidence recorded above this means, in our opinion, that the protein-nucleic acid ratios of the nuclei which we have prepared represent within narrow limits those which they possessed when intact in the cells from which they originated. It further means that the widely different values obtained by Pollister & Leuchtenberger are erroneous. It is, however, unnecessary to inquire in detail into the source or sources of their error, for Ris & Mirsky (1950), using, as did Pollister & Leuchtenberger, the apparatus designed by Pollister & Ris (1947), find that if they apply to their own results the methods employed by Pollister & Leuchtenberger for calculating the nucleic acid content of nuclei from measurements of their optical

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

densities due to Feulgen staining, the results are only 25 % of those obtained by direct chemical determination. If it is true, as this suggests, that Pollister & Leuchtenberger's nucleic acid values are only one-quarter of their correct values it is obvious that, after applying the necessary correction to their results, their protein-nucleic acid ratios would come into approximate concordance with ours. A misinterpretation by Pollister & Leuchtenberger of the value of Feulgen staining for quantitative work does not, however, appear to be the sole source of error in optical measurements of this kind, for Di Stefano (1948), working in Pollister's laboratory, determined the nucleic acid content of nuclei by measurements of optical densities in ultra-violet light and obtained values which were in complete agreement with those found with Feulgen staining. From investigations of various aspects of Feulgen's reaction (Stedman & Stedman 1948, 1950*b*; Ely & Ross 1949; Ris & Mirsky 1950), it is now certain that the technique used by Pollister & Leuchtenberger cannot possibly yield correct values for the nucleic acid content of cell nuclei, and it is therefore clear from Di Stefano's result that measurements made with ultra-violet light are also subject to large error.

Extraction of basic proteins from cell nuclei

It has been known since the original work of Miescher (1897) on salmon sperm heads and the extension of his methods to the nuclei of avian erythrocytes by Kossel (1884) that basic proteins can be extracted from certain isolated cell nuclei or from nuclear material by means of dilute mineral acids. This method has, in fact, usually been employed for the preparation of protamins and histones from fish sperm heads and of histones from nucleated erythrocytes. Nevertheless, the histone from the thymus gland of the calf, which is the only member of this group of basic proteins which has hitherto been submitted to any extensive chemical investigation, has always been prepared by other methods (Lilienfeld 1894; Felix & Rauch 1931) from the whole gland. The basic proteins used in the present investigation have, with the exception of that from wheat germ, been extracted from isolated cell nuclei. This procedure not only ensures that they do, in fact, originate from the nucleus, but it also constitutes an important step in their purification.

Histones

For the extraction of histones, 2 g. of dry cell nuclei are weighed into each 50 ml. centrifuge tube used. Since dry nuclei do not easily wet with aqueous solvents, they are stirred with a small amount of absolute alcohol until thoroughly wet with this solvent. The resulting paste is treated with excess of water and, after mixing, centrifuged. The clear supernatant liquid is rejected and the solid again brought into fine suspension in water and left overnight to soak in this solvent. The water is removed by centrifugation and the solid intimately mixed with 20 ml. of 0·1 N-sulphuric acid. After 20 min., during which time the mixture is frequently, although not continuously, stirred, the tube is again centrifuged and the clear, acid, supernatant liquid pipetted from the residue and treated with excess (about 40 ml.) of absolute alcohol. An immediate precipitation of the sulphate of the histone usually occurs, but as complete flocculation only takes place slowly the mixture is left overnight, or longer if necessary, for this to occur. Meanwhile, the residue is again similarly extracted with 0·1 N-sulphuric acid and the process repeated

until the acid extract fails to give a turbidity with excess of alcohol. About eight successive extractions are normally necessary to remove the histone completely, although the bulk of the product is contained in the first three extracts. The various fractions of the histone sulphate so prepared are collected together in the centrifuge and dried by two treatments with absolute alcohol and one with dry ether. The suspension in ether is then filtered at the pump, when the histone sulphate is obtained as a light, snow-white powder. The yield is calculated from the weight of the air-dry substance.

This extraction procedure has proved to be uniformly successful when applied to nuclei prepared from compact tissues, but difficulties have sometimes, although by no means invariably, been encountered when it has been used with fowl erythrocyte nuclei. In such cases treatment of the nuclei with water has caused the latter to coalesce to a sticky mass which is impenetrable to the acid added subsequently. When this has occurred, it has been necessary to dry the nuclear material with alcohol, which eventually causes it to set to a hard, brittle mass, and to resume the extraction of the latter with acid after grinding it to a powder. Losses of material necessarily occur during this treatment. For this reason, and because histones are not so readily extracted from such material as from a fine suspension of nuclei, a low yield of histone almost certainly results. The cause of this phenomenon is obscure. As far as we are aware, however, it has only occurred with preparations which have not been treated with 4 % acetic acid, which reagent no doubt exerts a slight fixative or coagulative effect on the nuclear contents. Such coalescence of the nuclei prepared by the saline treatment has not occurred when the material, wetted with alcohol, has been treated directly with the acid used for the extraction without first washing away the alcohol.

In most of our work the histone has been prepared in the above manner in the form of its sulphate. For some purposes it is convenient to prepare the chloride. This can be effected by the same procedure using 0·1 N-hydrochloric acid in place of the sulphuric acid and precipitating the chloride with acetone. The chloride is apparently much more soluble than the sulphate, and is not precipitated from aqueous solution by alcohol. When acetone is used for this purpose, a very large excess must be added. The chloride dissolves in water to give a clear, colourless solution, but if it is desired to reprecipitate it with acetone it is advisable, if the use of very large volumes of acetone is to be avoided, to add a moderate excess of hydrochloric acid to the solution. The solid chloride is dried by treating it twice with acetone and once with ether. It is filtered, as in the case of the sulphate, from the latter solvent at the pump.

Protamins

The two protamins, clupeine and salmine, present in the sperm heads of the herring and salmon respectively, can be extracted from these nuclei in the form of sulphate by the method used for histones. Alternatively, the extraction may be made with 0·1 N-hydrochloric acid and the sulphate precipitated from the extract by the addition of sulphuric acid as well as alcohol. As pointed out elsewhere (Stedman & Stedman 1947*b*), however, decinormal mineral acid, while removing a considerable proportion of the protamin from the sperm heads, does not remove it completely in ten extractions. Hence, for quantitative estimations of the amount present in the sperm heads we have employed

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

0·5 N-sulphuric acid, which removes the protamin so rapidly that only three extractions are found to be necessary. Whichever extraction procedure is employed, the protamin sulphate usually separates on the addition of alcohol as an oil. This is allowed to settle; the mother liquor is then decanted and the oily deposit stirred with absolute alcohol, when it solidifies. It is resuspended in alcohol and finally in ether, from which it is removed by filtration.

Basic proteins from nucleoproteins

Extraction of basic proteins from the nucleoprotein obtained from wheat germ has been effected with decinormal sulphuric acid in the manner described for histones. Owing to the great tendency of the nucleoprotein to clot to a mass when treated with acid, some difficulty has, however, been experienced in doing this. A similar difficulty has arisen in extracting histone from the nucleoprotein obtained by submitting minced calves' thymus glands to the treatment described for wheat germ. Our experience with this type of material has not been extensive, but the best results seemed to be obtained by stirring the nucleoprotein with the acid, allowing the solid to settle by gravity alone and, after removing the extract, repeating the process until no further basic protein is removed. The histone sulphate is then obtained from the extracts in the usual manner.

Basic protein content of cell nuclei

The yield of basic protein extracted from isolated cell nuclei by the above method is regarded as a measure of the histone content of the particular type of nucleus used. It obviously does not give an absolutely accurate value for, in most cases, it is impossible to correct the yield for the weight of sulphuric acid combined with the histone. Moreover, an isolation procedure can rarely be effected with quite the precision of a trustworthy method of analysis. For these reasons it has seemed sufficiently accurate to calculate the percentage content of basic protein in the cell nuclei which we have examined as the weight of air-dry sulphate isolated from 100 g. of air-dry nuclei. Provided the extraction has been complete and losses during precipitation and manipulation have been avoided, such a method should give a somewhat high value. Incomplete extraction of a sufficient magnitude materially to affect the result can, we believe, be excluded. In order, however, to provide a rough check as to the possibility of other losses occurring we have, in a few cases, determined the weight of the residue from the extraction of the histone after thoroughly washing it with water and drying it with alcohol and ether. This has shown that the sum of the weights of histone sulphate and residual nuclei is usually slightly smaller than the weight of the original nuclei. For example, 8 g. of thymocyte nuclei from the calf yielded 2·12 g. (26·7 %) of histone sulphate, while the residue weighed 5·76 g. (72 %). The sum of the two products thus amounted to 99 % of the weight of the nuclei taken. Another preparation of similar nuclei gave 27·3 + 73·2 % = 100·5 %. It follows from such results that the yield of the sulphate provides a reasonable and serviceable value for the content of histones in the cell nucleus. One slight discrepancy, which we cannot yet definitely explain, has, however, recently arisen. As will be seen below, when the histone is isolated in the form of chloride instead of sulphate the yield is slightly higher. That this difference is not due to a variation in the composition of the

nuclei has been shown in one instance by extracting equal portions of the same batch of nuclei with sulphuric and hydrochloric acid respectively. The yield of hydrochloride proved to be somewhat higher than that of the sulphate. One possible explanation of this result is that the chloride which precipitates contains more equivalents of anions than does the sulphate.

When determined in the above manner, the histone content of nuclei of the same type proves to be remarkably constant. In a score or more determinations on preparations of nuclei from different batches of calves' thymus glands values of from 27 to 29 % have been consistently obtained, although in one experiment in which the extraction was effected with hydrochloric acid, the yield of choride was 32 %. Nuclei from human thymocytes give almost identical values (Stedman & Stedman 1947*b*). Those from ox spleens, which are mainly derived from cells (small lymphocytes) which are morphologically identical with thymocytes similarly gave, in two cases, yields of 28·9 and 29·9 %, values agreeing with those previously found for lymphocytes from mice. There can thus be little doubt, particularly as the nucleic acid contents also correspond closely, that the nuclei of small lymphocytes and thymocytes from these three species of mammals possess compositions which are quantitatively identical, although some of the components doubtlessly differ qualitatively among the different species.

The nuclei from liver cells have not been investigated so extensively as have lymphocyte nuclei. Nevertheless, it is clear that they also possess a characteristic composition which differs from that of the latter both in histone and nucleic acid content. Thus, many preparations of nuclei from ox livers have given values of 23 and 28 % for histone and nucleic acid respectively. Two recent values for the histone content are 22·8 and 22·6 %. As pointed out on p. 572, however, the value for histone may rise to 25 % and that for nucleic acid to 30 % if the liver pulp is washed with saline before the nuclei are liberated. It is probable that the latter figures are the more accurate ones. Liver nuclei from the mouse resemble those from the ox in composition.

Preparations from fowl erythrocytes provide another example of nuclei of a given type possessing a characteristic composition. Six different preparations have been examined. Half of these were extracted with sulphuric acid and gave yields of histone sulphate of 24·2, 22·2 and 23·6 %. The remainder were extracted with hydrochloric acid and yielded 28·0, 25·3 and 29·7 % of histone chloride. The low value of 25·3 % for the chloride given by one preparation was due, as explained on p. 572, to the contamination of the nuclei with sodium chloride. In view of the discrepancy between the yields of sulphate and chloride, separate portions of another preparation of these nuclei were extracted with the two acids. They gave yields of 22·5 and 28·5 % for the sulphate and chloride respectively. From phosphorus determinations on four preparations of erythrocyte nuclei, the nucleic acid contents were calculated to be 37·1, 37·1, 38·1 and 37·8 %. Fowl erythrocyte nuclei thus resemble mammalian liver nuclei with respect to their content of histone and lymphocytes with respect to that of nucleic acid.

A knowledge of the basic protein content of cell nuclei is important not only because its value is usually constant for, and characteristic of, normal cells of a particular type, but also because it enables an estimate to be made of the extent to which other proteins enter into the composition of various cell nuclei. For this purpose it is also necessary to

know the nucleic acid content, a fairly accurate value for which can be calculated from a phosphorus determination on the lipid-free, dry nuclei. It was, in fact, by this method, combined with the demonstration of the presence in nuclei of the protein chromosomin, that we were able to show (Stedman & Stedman 1943) that cell nuclei, which had formerly been thought to be composed entirely of histone (or protamin) nucleate (see Stedman & Stedman 1947*a*) were, in fact, more complex and contained three main components: nucleic acid, basic protein and chromosomin. As pointed out by Schmitt (1944) at the time, this result was in direct conflict with the work of Mirsky & Pollister (1942, 1943), who adhered to the classical view of the composition of cell nuclei. Mirsky & Pollister (1947) and Mirsky & Ris (1948) have, however, since confirmed the presence of chromosomin in cell nuclei. Nevertheless, they still maintain that the nuclei of calf thymocytes contain over 90 % of histone nucleate. This conclusion is in such direct conflict with our results that it is necessary to consider the basis of their claim.

In their more recent work, Mirsky & Ris (1948) use material which they describe as 'chromosomes'. We do not share their view of the nature of this material. It contains, it is true, threadlike structures, but these are unlike any known chromosomes. Moreover, according to Calvet, Siegel & Stern (1948), who have made an electron microscopic study of the so-called chromosomes, they consist of 'random fragments of the chromatin network'. Lamb (1949), in similar studies, concludes that they are formed by drawing out the nuclei into thread-like formations which later break off as isolated threads. There is, however, evidence of an even more convincing nature that the thread-like structures are not chromosomes; they possess a composition which is to all intents and purposes identical with that of the intact nucleus from which they originated. This follows, in the case of thymocyte 'chromosomes', from their nucleic acid content. Thus, Mirsky & Ris (1948) state that their lipid-free 'chromosomes' from calf lymphocytes contain 37 % of their weight of nucleic acid. According to Stedman & Stedman (1947*b*) the lipid-free nuclei also contain 37 % of nucleic acid. Similarly, Mirsky & Pollister (1947) converted isolated thymocyte nuclei into nucleoprotein by dissolving them in molar saline and precipitating the nucleoprotein by dilution with water. This material again contained 37 % of nucleic acid. These results lead inescapably to the view that the 'chromosomes' are identical in composition with the nucleus itself. Ris & Mirsky (1949) have, indeed, been forced to the conclusion that their 'chromosomes' do, in fact, fill the whole nucleus, a conclusion which, in our view, constitutes a *reductio ad absurdum*. Evidently Mirsky & Ris's threads are not chromosomes. If they were, a dilemma would arise of the same nature as that caused by the classical view of the composition of cell nuclei, the resolution of which has been outlined elsewhere (Stedman & Stedman 1947*a*); this solution still holds.

Mirsky & Ris claim that their 'chromosomes', which, as we have seen, are identical in composition with the whole nucleus, contain 55 % of histone. With a nucleic acid content of 37 %, the nucleohistone content thus becomes 92 %. On the other hand, on the basis of our results the nucleohistone content should not exceed 70 %. There is obviously a big discrepancy between the two results. Mirsky & Ris suggest that this is due to the impossibility of extracting histone completely from isolated nuclei, a view which will be considered below. In our opinion, much of the discrepancy is due to the methods

used by these authors both for measuring and for calculating the histone content of their 'chromosomes'. To do this they dissolve them in molar saline and centrifuge the solution at high speed. This causes some chromosomin, contaminated or combined with a small proportion of nucleic acid, to sediment, but there is no evidence that its sedimentation is complete. They then ascertain the nucleic acid content of the nucleoprotein contained in the supernatant by precipitating it from an aliquot of the fluid and determining the phosphorus content of the dry material. The value obtained is 45 %. Histone determined in another aliquot colorimetrically is said to constitute 47 % of the mass of the fibrous nucleoprotein in the supernatant. The sum of the two components amounts to 92 %, and Mirsky & Ris therefore conclude that 8 % of the histone has been lost in the determination. If this is added to the 47 % estimated, a total of 55 % is obtained which, when added to the 37 % of nucleic acid originally present in the 'chromosomes', gives a total of 92 % of histone nucleate in the latter. It is, of course, clear that this figure is arrived at, not as a result of the estimation of histone, but largely on the basis of the weight (8 %) of chromosomin in the sediment produced by the centrifugation of the solution in molar saline. There is, moreover, an error in Mirsky & Ris's calculations. A value of 47 % histone in the material containing 45 % nucleic acid corresponds with a value for histone of 38 % in the original 'chromosomes', which contained only 37 % nucleic acid. The sum of the histone and nucleic acid contents of the original material thus becomes $38+37=75$ %, a figure which approximates to the 70 % maximum of our results. It should be noted that the Millon colour which Mirsky & Ris reported to be present on the precipitate which formed during the above estimation of histone, and which they attributed to histone, could well be due to chromosomin which had escaped sedimentation during centrifugation.

In view of the conflicting results yielded by our methods and those of Mirsky and his collaborators on the composition of the nuclei of calf thymocytes, it is of interest to note that something approaching agreement has been obtained with the nuclei from ox-liver cells. According to our results, and selecting maximum figures, these contain 25 % of histone and 30 % of nucleic acid, so that the amount of histone nucleate which it should be possible to obtain from them by Mirsky's method is 55 %. According to Mirsky & Ris 'chromosomes' from calf livers contain 45 % of histone nucleate. Evidently our method of extraction has, in this case, given quantitative yields of histone. There is thus no reason why it should not also do so, as we are confident it has done, with the nuclei from thymocytes.

As pointed out above, Mirsky & Ris consider that it is impossible to extract histones quantitatively from cell nuclei. There is a considerable discussion on this subject in the older literature culminating in the failure of Steudel & Peiser (1922) to extract histone quantitatively by means of dilute mineral acid from histone nucleate prepared from the two components, and hence known to contain only these components. The explanation of this failure is simple and of a purely physical nature. The nucleic acid, liberated by the mineral acid from its combination with histone, immediately forms a sticky mass through which the solvent is unable to diffuse. Hence complete extraction of the histone is impossible. Cell nuclei do not, however, consist solely of histone or protamin nucleate, although this view obtained at the time Steudel made his experiments. They also contain

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

chromosomin, and there is little doubt that the difficulties experienced in extracting histone or protamin from the nucleates of these bases are minimized in cell nuclei because the nucleic acid, liberated from its combination with the base, then combines with chromosomin. Nevertheless, as the experiments described above show, difficulty is sometimes encountered with cell nuclei, but this has only occurred with nuclei which have not been submitted to the 4 % acetic acid treatment. With such nuclei the extraction of histone proceeds smoothly and without difficulty.

Purification of histone and protamin sulphates

Histones

The sulphates of the histones prepared by the above method from various cell nuclei were, in our earlier experiments, purified by dissolving them two or three times in water acidified with a small amount of dilute sulphuric acid, centrifuging the material to remove any insoluble material and then reprecipitating the histone sulphate with alcohol. Table 1 gives some analyses, which are useful for comparison with later figures, made on histones from various sources purified in this way. The results at first suggested that the histones present in the thymocytes, lymphocytes and liver cells of the ox were, in fact, different substances. But when the tyrosine contents of numerous preparations of thymus histone sulphate from the calf were determined, discordant figures were obtained. The tyrosine

Table 1. Analyses of some histones before fractionation

species	source	salt used	N-content (%)	amide-N (% of total N)	arginine-N (% of total N)
ox	thymus	sulphate	16·1	4·69	26·1
ox	spleen	sulphate	16·3	5·00	28·8
ox	liver	sulphate	16·7	5·11	30·8
fowl	blood	sulphate	15·6	4·68	28·6
fowl	blood	chloride	17·1	5·77	28·6

contents, expressed as a percentage of the dry weight of the sulphate, varied from 3·5 to 4·0 %. It seemed clear from these results that a partial fractionation was being effected by reprecipitation, which had been carried out solely with a view to removing traces of insoluble material, and that this fractionation had proceeded to different degrees in the various preparations. One specimen of thymus histone sulphate was therefore separated into three fractions by precipitating it from aqueous solution by the successive addition of suitable volumes of alcohol. Some difficulty was experienced in doing this, for when a solution of histone sulphate is treated with an amount of alcohol insufficient to cause almost complete precipitation, the fraction which separates retains much water and consequently forms a light, jelly-like precipitate which is difficult to separate from the liquid by centrifugation. The three fractions which were eventually obtained were dried with alcohol and ether and analysed for their contents of arginine and nitrogen. Two of them were also analysed for tyrosine. The results are given in table 2. These left no doubt that the histone was not homogeneous. The following method of purification, based on its fractional precipitation with alcohol, was therefore devised.

Each gram of crude histone sulphate, as isolated directly from cell nuclei, is placed in a centrifuge tube and dissolved in about 40 ml. of water by rubbing it with a glass rod

in the tube, a process which frequently takes about an hour owing to the sticky mass which the histone forms on being first wetted with water. This solution is then centrifuged and the clear, colourless supernatant liquid pipetted from a small sediment of insoluble material into a flask. The centrifuge tube and the sediment which it contains are washed with about 15 ml. of water, the tube again centrifuged and the washings united with the

TABLE 2. FRACTIONATION OF CRUDE THYMUS HISTONE SULPHATE FROM THE CALF

fraction	total-N (%)	amide-N (% of total N)	arginine-N (% of total N)	tyrosine (%)
most soluble	15·96	4·14	18·3	1·78
middle	17·00	5·13	28·7	4·26
least soluble	17·00	4·96	30·3	—

main solution. To this is added, first, one drop of bench dilute sulphuric acid and then, slowly and with constant shaking, absolute alcohol until the solution becomes markedly opalescent. Further small additions of alcohol are then made at short intervals until a slight but perceptible precipitate forms. The solution is left overnight, during which time the histone sulphate settles as a bulky mass. The contents of the flask are transferred to centrifuge tubes with as little disturbance as possible and centrifuged. If the mixture has been shaken too vigorously during transference, centrifugation sometimes presents difficulties. Otherwise, the precipitate collects as a bulky, opalescent jelly-like mass at the bottom of the tubes with a clear solution above. The latter is removed with a pipette and the jelly intimately mixed with absolute alcohol; this causes a considerable contraction of the material, no doubt due to the removal of water. After about an hour the tube is centrifuged, the supernatant liquid removed and the sediment again stirred with absolute alcohol. Dehydration is allowed to proceed for some hours, usually overnight, when the alcohol is removed by centrifugation and the pasty residual sediment stirred with dry ether. This transforms the sediment into a light, flocculent solid which is filtered with suction and carefully washed with dry ether. The whole operation is then repeated three more times on the solid. This procedure has been applied successfully to the crude histone sulphates isolated from cod sperm heads and the nuclei from calves' thymus glands, ox livers, ox spleens, fowl erythrocytes, fowl thymus glands, fowl spleens, salmon livers and human glands, spleen and tumour. The yields of the main products at each stage of the fractionation and the percentage yield of the final preparation are recorded in table 3. The figures are of interest in several respects. The loss of weight is so much greater at the first than at subsequent precipitations that it can be assumed that the fractionation is almost completed during the first two stages. Different preparations of crude histone obtained from nuclei of the same type give similar yields of the purified substance, thus demonstrating the uniformity of the fractionation process and suggesting that the impurity removed is a constant component of the nuclei and not an adventitious impurity. In general, this yield is also fairly constant for different types of nuclei from the same or different species, although there is one important exception. The crude histone isolated from fowl erythrocyte nuclei gave a yield of the purified histone which is definitely smaller than those from lymphocytes and thymocytes of the same species, a result which corresponds with the presence, as described on a later page, of a histone in the mother liquors from the erythrocyte histone which is absent from those of the lymphocyte and

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

thymocyte histones. The slight difference between the yields from calf thymocyte and ox-liver nuclei is probably not significant, but it is mentioned here because it nevertheless conforms, as a comparison of the results in tables 1 and 4 will show, with the greater change produced by fractionation in the composition of thymus than of liver histone.

TABLE 3. YIELDS OF MAIN FRACTION OF HISTONE SULPHATE DURING FRACTIONATION

species	source	weight of crude histone sulphate (g.)	yield (g.) from fractionation no. 1	2	3	4	final yield (%)
ox	thymus	4	3·31	3·18	2·94	2·84	71
ox	thymus	4	3·30	3·15	—	2·79	69
ox	thymus	4	3·22	2·98	2·83	2·77	69
ox	thymus	4	3·27	3·02	2·86	2·71	68
ox	thymus	2·93	2·56	2·31	2·17	2·12	72
ox	spleen	1·99	—	1·42	1·38	1·28	64
ox	spleen	1·96	1·64	1·54	1·45	1·42	72
ox	liver	4	3·50	3·28	3·12	3·09	77
ox	liver	4	3·38	3·27	3·10	3·04	76
fowl	blood	2·09	1·32	1·13	—	0·99	47
fowl	blood	4	2·65	2·31	—	2·00	50
fowl	thymus	1·89	1·61	1·52	1·50	1·45	77
fowl	spleen	1·15	0·97	0·90	0·88	0·86	75
cod	sperm	1·90	1·59	1·50	1·36	1·33	70
cod	sperm	3·33	2·81	2·53	2·32	2·29	69
salmon	liver	1·45	1·25	1·15	1·07	1·03	71
man	spleen	2·00	1·67	1·60	1·54	1·46	73
man	lymph glands	2·08	1·78	1·63	1·55	1·51	73
man	broncogenic carcinoma	0·76	0·64	0·60	—	0·47	62
wheat	germ	2·00	1·50	1·34	1·21	1·09	54

The histones purified in the above manner will be referred to as the main histones of the cell nuclei from which they originate. This will distinguish them from the more soluble basic proteins, which will be provisionally termed subsidiary histones, remaining in the mother liquors.

Protamins

The two protamins, clupeine and salmine, which have been examined are so similar in properties that identical methods have been used for their purification. Thus, 1 g. of crude clupeine sulphate was dissolved in 20 ml. of water, the solution centrifuged to remove a small amount of insoluble material and the supernatant liquid treated with 5 ml. of alcohol. The colourless, oily precipitate which formed was centrifuged down and immediately stirred with alcohol, which caused it to solidify rapidly to a hard, white solid. This was then submitted twice more to the same treatment, when about 0·8 g. of the final product, known as 11V*b*1, was obtained. After the first precipitation, the product, presumably as a result of the loss of sulphate radicals, diminished in solubility, thus necessitating the use of larger volumes of water to effect solution. For reprecipitation, 5 ml. of alcohol per 20 ml. of solution was consistently used.

For comparison with the above product, 1 g. of the same crude preparation was dissolved in water and reprecipitated once only. The yield of product, which was termed V*a*, was 0·96 g.

Salmine sulphate was reprecipitated four times in a similar manner, the final preparation being known as 111X1.

Methods of analysis

General

The analytical procedures described in this section were carried out on material dried to constant weight in a high vacuum over phosphorus pentoxide at room temperature. Nitrogen was determined by micro-Kjeldahl and phosphorus by the method described elsewhere (Stedman and Stedman 1950*b*).

Arginine

The method employed for the estimation of arginine in proteins has been used in this laboratory since 1943. Meanwhile its essential features have been described by Macpherson (1946), although details of its application in the two laboratories differ. The method is based on the observation of Plimmer & Rosedale (1925) that, with certain reservations as to the existence of other possible sources of ammonia, arginine can be determined in protein hydrolysates by measuring the ammonia produced by their alkaline hydrolysis. Plimmer & Rosedale worked on a macro scale. We, like Macpherson, have adapted the process to a micro method. The apparatus for the alkaline hydrolysis is exceedingly simple and can be readily constructed from an ordinary micro-Kjeldahl apparatus by interposing a small reflux condenser between the head of the Kjeldahl flask and its normal condenser. The hydrolysis is then effected in the flask with the whole apparatus assembled as for a normal Kjeldahl estimation. When hydrolysis is complete, the water is run out of the reflux condenser and the ammonia blown over with steam in the customary manner. The details of its application to histones are as follows.

The histone sulphate (about 220 mg.) or protamin sulphate (about 150 mg.) is hydrolyzed by refluxing it with 7 ml. of 7N-hydrochloric acid for 30 hr. Without removing the acid, the hydrolysate is made up to 25 ml. with water, and 1, 10 and 5 ml. (2 ml. in the case of protamins) aliquots of this solution are used respectively for the determination of total nitrogen, 'amide' nitrogen and arginine nitrogen. With the exception of the 'amide' nitrogen, these are made in duplicate.

'Amide' nitrogen is determined in the ordinary micro-Kjeldahl apparatus. To the 10 ml. of hydrolysate contained in the flask is added 1 g. of calcium hydroxide made into a paste with a little water. Steam is then passed through the apparatus in the usual way.

For the arginine nitrogen, 5 ml. of the hydrolysate (or 2 ml. hydrolysate plus 3 ml. water) are placed in the flask of the modified Kjeldahl apparatus and 8 ml. of 50 % sodium hydroxide, followed by 3 ml. of water, added. The mixture is then boiled under reflux for 6 hr., when the ammonia formed is collected by steam distillation. This ammonia represents 50 % of the arginine nitrogen plus the amide nitrogen. The total arginine nitrogen is therefore twice the difference between these two values.

In testing the hydrolysis procedure with a good specimen of arginine mono-hydrochloride, the following results were obtained: total N, 25·8; arginine-N, 25·2; the theoretical nitrogen content being 26·6 %. The specimen thus contained 97·0 or 94·7 % of arginine hydrochloride according to the respective determinations with the Kjeldahl or hydrolysis procedures. If the impurity indicated by the total nitrogen content to be present in the arginine was non-nitrogenous, it is evident that, as Macpherson claims,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

the recovery of arginine by the hydrolysis procedure, which in the above experiment amounts to 97·7 %, is not complete. Estimations made on proteins would, in fact, require correcting by the addition of 2·3 % to the determined values. We are, however, loth to apply such a correction for the following reasons. Determination of the arginine content of salmine gave an uncorrected value, expressed as the percentage of the total nitrogen due to arginine, of 88·9. If this were increased by 2·3 %, it would become practically 91, a value which is, we believe, higher than any recorded value. Thus, recent values from the literature are: 90·2 (Block & Bolling 1945*b*), 86·5 (Macpherson 1946) and 89 (Tristam 1947). While this result gives some support to the view that the impurity in our specimen of arginine is nitrogenous it is by no means conclusive. There is, moreover, a further complication due to the possibility that amino-acids other than arginine may contribute a small quota of ammonia during the hydrolysis. We have therefore deemed it preferable to quote uncorrected results. As those obtained with salmine show, these must give substantially, if not absolutely, correct values for arginine, and we have been less concerned in this investigation with absolute values for particular amino-acids than with a method of estimation which can be carried out with precision, will yield reproducible results and will therefore serve admirably for the characterization and comparison of histones from different sources. The method which we have described meets these requirements.

Tyrosine

This was determined colorimetrically with the aid of the Millon reaction, using a procedure which, in the main, follows that described by Block & Bolling (1945*a*). The exact details are as follows. The histone sulphate (about 6 mg.) contained in a small flask with a 10 ml. graduation mark on the neck is dissolved in 0·4 ml. of water, a process which is conveniently allowed to complete itself overnight. This treatment has the double object of ensuring the dissolution of the histone, which otherwise causes difficulty, and of compensating for the evaporation of water during the subsequent hydrolysis. To this solution is added 0·5 ml. of 6 N-sodium hydroxide, when the mixture is heated on a boiling water-bath without any arrangement, apart from the short neck of the flask, for preventing evaporation. After 5 hr. the flask is cooled and its contents acidified with 0·7 ml. of 7 N-sulphuric acid. Two ml. of 15 % mercuric sulphate in 5 N-sulphuric acid are then added, when the flask is heated for 10 min. on a boiling water bath. After cooling, 1 ml. of 7 N-sulphuric acid, 2 ml. of 0·2 % sodium nitrite and water to the 10 ml. mark of the flask are then added, and the optical density of the solution measured exactly ten minutes later in a Spekker absorptiometer using a spectrum blue filter. The amount of tyrosine present is read from a calibration curve, which is checked for each determination by means of a standard containing approximately the same amount of tyrosine as the unknown. No correction has been made for the destruction of tyrosine during the hydrolysis.

Analyses of main histones and protamins

Histones

The analytical results given by the main histone sulphates from various species and organs are given in table 4. Where complete duplicate analyses are recorded, they have been made on histones prepared from entirely different batches of cell nuclei. This has

been done in as many cases as practicable. With the exception of the 'amide' nitrogen, for which only one determination has been carried out, all estimations have been made at least in duplicate. Individual figures have, however, only been quoted when replicate figures did not agree exactly.

TABLE 4. ANALYSES OF SULPHATES OF MAIN HISTONES

species	source	N content (%)	amide-N (% of total N)	arginine-N (% of total N)	tyrosine (%)
ox	thymus	17·0	4·95	29·8	4·37, 4·42
ox	thymus	17·0	5·19	29·1	4·32, 4·40 4·29, 4·32
ox	spleen	17·6	4·77	29·3	4·33, 4·25
ox	spleen	17·0	5·00	29·7	4·31, 4·41 4·35, 4·36
ox	liver	16·8, 16·7	4·99	30·4	4·32, 4·46
ox	liver	17·3, 17·2	4·93	29·5	4·45, 4·58, 4·47
fowl	blood	17·1	5·35	26·0	4·41, 4·43
fowl	blood	16·5	5·24	26·9, 26·5	4·32, 4·44
fowl	spleen	16·5	5·28	29·8	3·92, 4·34
fowl	thymus	16·6	5·07	29·4, 29·2	4·32, 4·30
cod	sperm	16·8	4·83	28·5, 28·8	3·31, 3·21
salmon	liver	16·9, 16·8	4·94	30·2	4·48, 4·51
man	broncogenic carcinoma	16·8	4·93	29·2, 29·8	4·41, 4·36
man	lymph glands	16·7	5·10	28·5, 29·3	4·60, 4·44
man	spleen	16·7, 16·9	4·74	30·6	4·38, 4·38
wheat	germ	15·1	4·23	19·5, 20·5	2·92, 2·90

In considering the bearing of the figures in table 4 on the question of the specificity of histones, two types of this phenomenon must be distinguished: species specificity and cell specificity. The former, when applied to homologous proteins, is a general phenomenon. It is exemplified in connexion with the basic proteins of cell nuclei by the protamins, salmine and clupeine, present respectively in the sperm heads of the salmon and herring, and the histone present in the sperm heads of the cod. These three proteins are clearly homologous proteins for they occur in the nuclei of similar cells and must therefore possess identical functions. The two protamins resemble one another closely in composition and were, in fact, at one time, thought to be identical. It is, however, now certain, as will be shown on p. 592, that they possess different compositions despite the small differences between the analytical results recorded in table 5. That the homologous histone of cod sperm is a different protein from the protamins has, owing to its marked difference in chemical and physical properties, never been in doubt. Species specificity of the basic proteins of cell nuclei is thus demonstrable in the sperm heads of these fishes, and there can be little doubt that it extends to the histones of other cell nuclei. Nevertheless, if the analyses for homologous histones in table 4 are compared, it is evident that they give no indication of species specificity in this sense. As far as the analyses have extended, it is impossible to distinguish between the histones from ox and salmon livers or between those from lymphocytes (or thymocytes) from the ox, fowl and man. It would be similarly impossible to distinguish between salmon and herring protamins on the basis of the small differences in their arginine contents given in table 5 were it not that other differences

in their composition are present. It is thus clear that, except in favourable cases, species specificity in the basic proteins of cell nuclei is difficult to detect by purely chemical means, although it must be assumed to exist.

The demonstration of cell specificity in basic proteins would, *a priori*, be expected to present more difficulties. In fact, its detection seems to depend, as does that of species specificity, on the choice of favourable material. When a protamin is present in the sperm head, as it is in the salmon, its difference from the histones present in the nuclei of the somatic cells is so obvious that it provides a clear-cut case of cell specificity which is as definite as that of the species specificity shown by a comparison of the basic proteins in salmon and cod sperm heads. On the other hand, as a reference to table 4 will show, there is little, if any, difference between the analytical results yielded by the histones from different cell nuclei of the ox. The same analytical methods, however, if applied alone, would scarcely serve to detect species specificity in salmon and herring protamins. Hence the results with histones from the ox must not be taken to disprove the existence of cell specificity in this species; they must be regarded as negative. The histones from the fowl present a much more favourable case. Those from the spleens and thymus glands, assuming them to originate from the nuclei of identical cells, would be expected to be the same, as in fact the analyses indicate, but do not prove, that they are. But the histone from the erythrocytes is definitely different in composition. Its arginine content is smaller than that of the lymphocyte histone. The difference, although not very large, is certainly outside the limits of experimental error. The result is, moreover, supported by the fact that in the purification of the total histone isolated from fowl erythrocyte nuclei, which has an arginine content (table 1) identical with that of purified fowl thymus histone, a subsidiary histone with a higher arginine content (table 7) is removed from it. Incidentally, this subsidiary histone is absent from thymocyte and lymphocyte nuclei and hence represents another example of cell specificity among the histones. Cell specificity of the basic proteins of cell nuclei has thus been demonstrated to occur between the sperm and somatic cells in the salmon, and between the erythrocytes and the lymphocytes in the fowl. It also almost certainly exists, as pointed out in the introduction, between the erythrocytes and the liver cells of the salmon. It is thus probable that it is a general phenomenon.

Protamins

Analyses of salmine and clupeine sulphates, before and after fractionation, are shown in table 5. It is clear that fractionation produces a slight change in composition. Its magnitude is, however, so small that it is evident that the basic material originally isolated from the sperm heads consisted to a preponderating extent of protamin. This is illustrated in a marked manner in the case of clupeine. The preparation V*a* was obtained in a yield of 96 % by fractionation of the unpurified product. Despite this high yield, two additional fractionations, giving preparation 11 V*b* 1, produced no further change in composition. Nevertheless, as will be described on p. 588, the mother liquors from the purification of both clupeine and salmine yielded a small amount of protein of a different character from the protamin.

The demonstration that the unpurified protamin consisted essentially of one substance of high arginine content is of interest because, while the homogeneity of salmine has, as

far as we are aware, never been questioned, this is not the case with clupeine. Following the work of Kossel (1928), it has been generally accepted that clupeine is a mixture of protamins with varying arginine contents. Our results do not support this view. The discrepancy is probably due to the use by Kossel of impure sperm heads, and possibly also to his use of hot solutions in the purification of his product, with the consequent contamination of the protamin with non-nuclear proteins and with its own partial decomposition products.

TABLE 5. ANALYSES OF SULPHATES OF PROTAMINS

species	preparation	N content (%)	amide-N (% of total N)	arginine-N (% of total N)
salmon	unpurified	22·9	0·96	87·7
salmon	111X1	24·1	0·69	88·9
herring	unpurified	22·9	0·82	86·2
herring	V*a*	24·2	0·78	87·6
herring	11V*b*1	24·1	0·65	87·9

SUBSIDIARY HISTONES

Evidence for the existence of subsidiary histones

The addition of small volumes of alcohol to the clear, colourless mother liquors obtained during the purification of the sulphates of the main histones produces no change in their appearance, but if a large excess of alcohol is added the liquid becomes intensely opalescent and, on standing, slowly deposits a white solid, a process which can usually be much accelerated by vigorously swirling the opalescent solution in the flask. When, as judged by the disappearance of the opalescence, precipitation is complete, the suspension is centrifuged and the sediment dried with alcohol and ether in the usual way. The product, while not deliquescent in the ordinary meaning of the term, tends to take up moisture from the air and to become sticky during filtration at the pump, although this can be prevented if due care is exercised. It is also much more readily soluble in water than is the main histone. Its properties thus clearly differ from those of the latter. Nevertheless, it is by no means a pure substance and is almost certainly contaminated with the main histone. In an attempt to remove this, the material so obtained was dissolved in water, the solution acidified with a drop of dilute sulphuric acid and then treated drop by drop with alcohol until a faint but permanent turbidity was produced. After a time a flocculent

TABLE 6. ANALYSES OF PARTLY PURIFIED SUBSIDIARY HISTONES

species	source	N content (%)	amide-N (% of total N)	arginine-N (% of total N)	tyrosine (%)
ox	thymus	15·8, 16·0	3·38	11·5	1·40
ox	spleen	15·7	3·09	13·3	1·29
ox	liver	15·9	3·27	13·8	1·70
fowl	thymus	15·2	3·36	20·3	1·48
cod	sperm	15·2	3·20	17·3	0·81, 0·86
wheat	germ	15·2	3·00	16·2	2·54

solid usually separated, although occasionally the liquid set to a gel. In the latter case the process was repeated with a more dilute solution, when a precipitate formed normally. The precipitate was removed in the centrifuge and the liquid treated with an excess of alcohol. This precipitated the bulk of the material, which was collected and dried by the

usual procedure. The analyses of such products, recorded in table 6, provides evidence, as a comparison with table 4 will show, that the mother liquors from the purification of the main histones contained one or more proteins which differ markedly in composition from them. Similar products were obtained during the fractionation of the histones from human tissues, fowls' spleens and salmon livers, but the amount of material available in these cases was too small to warrant further examination. On the other hand, the total histones from calves' thymocytes and fowls' erythrocytes are more readily accessible. They have therefore provided material for a somewhat more detailed examination of the nature of the products obtained in the manner described above. The results, taken in conjunction with the analyses in table 6, seem to leave no doubt that the proteins associated with the main histones in acid extracts of isolated cell nuclei are basic proteins and can therefore be regarded as subsidiary histones.

It is probable, although not yet certain, that a protein of the nature of a subsidiary histone is associated with the protamins present in fish sperm heads. Thus, when treated with an excess of alcohol, the mother liquors from the fractionation of 2 g. of salmine sulphate yielded a small amount of precipitate. This was dissolved in 20 ml. of water and treated with 4 ml. of alcohol. An oily precipitate, presumably consisting of protamin sulphate, separated. This was centrifuged down and rejected. Addition of excess of alcohol to the supernatant then gave a precipitate which, on drying, weighed 100 mg. This, known as X2, was used for chromatographic analysis, as will be described on a later page. A similar fraction was obtained from the mother liquors from the fractionation of clupeine. The amounts of these substances obtained was, however, too small for further examination.

Fractionation of the subsidiary histones from fowls' erythrocytes

Corresponding with the smaller yield of main histone from the total histones extracted from the nuclei of fowls' erythrocytes (see table 3), a larger yield of subsidiary histone is obtained from this than from similar material from other tissues and species. Thus, in one experiment, 2·09 g. of total histone sulphate yielded on fractionation 0·85 g. of crude subsidiary histones. The fractionation of this material, which was termed 2F2, is best described by the use of our serial numbers. 2F2 was dissolved in water, one drop of dilute sulphuric acid was then added, as in all subsequent fractionations of this material, and the solution treated with sufficient alcohol to produce an opalescence but no immediate precipitate. On standing overnight, a considerable precipitate formed. This was centrifuged yielding a solid 12F2. The supernatant liquid was treated with more alcohol, which caused a further precipitate 22F2 (0·28 g.) to form. Without drying, 12F2 was dissolved in water and alcohol added drop by drop until the solution was slightly turbid. The precipitate, 112F2, which formed was removed and more alcohol added to the mother liquor giving the precipitate 212F2 (0·21 g.). Again without drying, 112F2 was dissolved in water and the solution carefully treated with alcohol until just turbid, the slight precipitate which ultimately formed being rejected. The mother liquor gave on the addition of alcohol 2112F2 (0·24 g.). The three fractions thus obtained accounted for nearly 86 % of the original material. They were analyzed with the results given in table 7.

While it is probable that none of these fractions was pure, a comparison of the results obtained with fractions 212F2 and 2112F2 indicates that the latter was approaching

purity. Unfortunately, through an accident, its tyrosine content was not determined exactly, but it was roughly estimated to be of the same order as that of 212F2. In any case, it is clear from the results that one of the subsidiary histones in the nuclei of fowl erythrocytes contains a greater proportion of arginine than does the main histone. Corresponding with this finding, and therefore confirming it, the total, unfractionated histone itself contains more arginine than does the purified, main histone, as a comparison

TABLE 7. ANALYSES OF FRACTIONS OF SUBSIDIARY HISTONES FROM THE FOWL

serial no.	N content (%)	amide-N (% of total N)	arginine-N (% of total N)	tyrosine (%)
22F2	15·6, 15·7	2·93	16·93	1·10
212F2	17·2	3·48	32·1	2·60
2112F2	17·5, 17·4	3·48	34·0	

of the results in tables 1 and 4 will show. The high arginine content of this basic protein and its nuclear origin justify its designation as a histone. Since, moreover, material with a similar high arginine content was absent from the subsidiary histone fraction from the thymocytes of the fowl, the presence of this histone in fowl erythrocyte nuclei represents a marked difference in chemical composition between two types of nuclei from the same species. The fraction 22F2, which represents the most soluble fraction of the subsidiary histones from fowl erythrocytes, contains less arginine and less tyrosine than either the subsidiary histone 2112F2 described above or the main histone (table 4). We regard this as evidence of the presence in fowl erythrocyte nuclei of at least one other subsidiary histone differing from the one described above in containing a lower arginine content than the main histone.

Fractionation of the subsidiary histones from calves' thymocytes

The crude subsidiary histones, weighing 3·2 g., which were available for this fractionation were obtained at various times from about 20 g. of total histone sulphate isolated from nearly 70 g. of dry nuclei from calves' thymocytes. The material, known as 2R2, was dissolved in about 60 ml. of water and the solution acidified with dilute sulphuric acid, a procedure which has been carried out at each stage of the fractionation. Attempts to precipitate from this solution a small fraction of the material by the careful addition of alcohol were unsuccessful; too much precipitate formed. More water was therefore added to bring the material already precipitated again into solution. Addition of alcohol then gave a precipitate 2R21 (2·02 g.). The mother liquors yielded with excess alcohol fraction 2R22 (0·98 g.). The less soluble of these two fractions, 2R21, was dissolved in 50 ml. of water and fractionally precipitated with alcohol, yielding a less soluble fraction 12R21 (0·7 g.) and a more soluble one 22R21 (1·26 g.). The subsequent fractionation of 12R21 need not be described in detail. It will suffice to say that it ultimately yielded two fractions with tyrosine contents of 1·73 and 1·37 % respectively for the less and more soluble ones. Fractions 22R21 and 2R21 were united and termed R3. Fractionation of this in the usual manner gave R31 (1·25 g.), tyrosine content 1·08 %, and R32 (0·92 g.), tyrosine content 0·38 %. R31 then yielded 1R31 (0·32 g.) and 2R31 (0·68 g.). The two more soluble fractions R32 and 2R31 were, apparently unfortunately, united and termed R4 (1·6 g.). Fractionation of this gave R41 (0·67 g.) and R42 (0·81 g.) with

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

a tyrosine content of 0·39 %. The more soluble fraction obtained by refractionation of R41 had a tyrosine content of 1·1 %. Refractionation of R42 gave, finally, 1R42 (0·22 g.), tyrosine content 0·55 %, and 2R42 (0·52 g.), tyrosine content 0·31 %. The latter fraction was probably not homogeneous, but further fractionation by the above methods seemed impracticable. In view of the very low tyrosine content of 2R42 it is not excluded that this amino-acid was due to the presence of an impurity, possibly the main histone, and that the protein of which 2R42 is mainly composed is free from tyrosine. In any case, 2R42 consists essentially of a protein quite different in its composition from the main histone, as the following analysis shows: N content, 14·35, 14·46 %: amide-N, 1·99 % of total N: arginine-N, 8·05 % of total N. Despite its low arginine content, we nevertheless class this protein as a histone because it is a basic protein which originates from the cell nucleus. That it possesses basic properties is shown both by the method used for its extraction from the nucleus and by the presence of sulphate ions in the hydrolysate used for the arginine estimation. As will be shown below, its basic properties are largely due to a high content of lysine.

The amino-acid composition of histones and protamins

The amino-acids of which the various histones examined are composed have been determined as far as possible by paper chromatography, using a procedure almost identical with that originally described by Consden, Gordon & Martin (1944). When sufficient of the hydrolysate prepared for the arginine determination was available, this was used for the chromatographic analysis. Alternatively, 10 mg. of the histone sulphate were hydrolysed under conditions similar to those employed for the larger quantities. One ml. of the larger, or the whole of the smaller, hydrolysate was evaporated *in vacuo* on a water-bath almost to dryness, the residue twice redissolved in water and again evaporated, and the final residue taken up in a small volume of water and 'desalted' electrolytically (Consden *et al.* 1947) to remove the last traces of hydrochloric acid. The residue from the evaporation of the solution of amino-acids so obtained was then dissolved in 0·4 ml. of 70 % aqueous alcohol and 0·01 or 0·015 ml. of this used for each chromatogram. Two-dimensional chromatograms were finally adopted using Whatman's no. 4 paper and selecting phenol and collidine as the most useful solvents. In some of the early experiments, phenol removed a considerable amount of brown impurity from the paper, which caused a haphazard movement of the fast-running amino-acids. Spraying the entire breadth of the paper with the phenol solvent at a level immediately below the point of application of the amino-acid solution, and for a depth of about 4 in., immediately before the development of the chromatogram with this solvent diminished, but did not entirely abolish, this difficulty when the two solvents were used in the order collidine-phenol. By reversing this order, however, spraying became entirely effective. The chromatogram was run first in phenol for about 20 hr. in a cabinet containing, in addition to the normal solvents (phenol and water containing sodium cyanide), a few drops of 0·88 ammonia in the tray beneath the paper. During this time, much of the brown impurity had accumulated in the vicinity of the advancing front and had reached the bottom of the paper. After drying the paper, development was carried out with collidine, with a few drops of diethylamine in the tray, for nearly 3 days. The slow move-

ment of the amino-acids, which rendered necessary this long period of development, proved to be an advantage, for the impurities which the solvent dissolved out of the paper were carried far in advance of the amino-acids and, together with the accumulated brown material from the phenol run, were removed entirely from the paper in the drops of solvent which fell into the tray after the advancing front had reached the end of the paper.

The chromatograms obtained from the hydrolysates of the main histones from calf thymus gland, ox spleen and liver, salmon liver, cod sperm and a human tumour were indistinguishable from one another. Figure 3, plate 20, which reproduces the chromatogram obtained from the main histone from cod sperm, thus serves to illustrate this group. Those from the main histones of fowls' erythrocytes, thymocytes and lymphocytes were essentially similar except for the absence of phenylalanine. The subsidiary histone with a high arginine content obtained from the nuclei of fowls' erythrocytes was indistinguishable qualitatively from the main histones of this species. On the other hand, the subsidiary histone with a low arginine content obtained from calves' thymocytes gave a chromatogram, reproduced in figure 4, plate 20, which not only differs qualitatively from that of the main histone by lacking phenylalanine, tyrosine and histidine and containing methionine, but also indicates a marked quantitative difference in amino-acid composition. As is evident from figure 4, the low arginine content of this histone is compensated for by a high lysine content, to which its basic properties must be largely due. It also differs from the main histone in containing, relatively to the other amino-acids, high amounts of alanine and proline.

From the above results it is evident that the main histones from cod sperm, salmon liver, calves' thymus glands, ox spleen, ox liver and a human tumour resemble one another in containing the amino-acids aspartic and glutamic acids, glycine, serine, alanine, threonine, proline, valine, the leucines, tyrosine, phenylalanine, arginine, histidine and lysine in much the same relative proportions, while the histones, including the subsidiary histone with a high arginine content, from fowls' erythrocytes, thymus glands and spleens are distinguished from these by the absence, at any rate from the chromatogram, of phenylalanine. Of the amino-acids which cannot be detected readily in the chromatograms of acid hydrolysates, tryptophan has been shown by separate tests (Stedman & Stedman 1947*b*) to be completely absent from all the histones, main or subsidiary, examined in this investigation. The question of the sulphur containing amino-acids has not yet been fully resolved. Felix & Rauch (1931) found the high content of 1·87 % of sulphur in the histone chloride which they isolated from the whole thymus gland of the calf. They also stated that the unpurified material, which they regarded as the native protein, gave a nitroprusside reaction for sulphydryl groups, but that this disappeared when it was purified through the picrate. We have examined the main histones prepared in this investigation for the presence of free sulphydryl groups by dissolving 20 mg. of the histone sulphate in 1 ml. of water and treating the solution with three drops of freshly prepared 5 % sodium nitroprusside and three drops of 10 % sodium cyanide. An immediate magenta colour was given by every main histone with the possible exception of that from wheat germ, which gave a faint pink colour. It thus seems certain that cysteine is present in the main histones. So far, however, we have obtained no evidence of the presence of methionine. Its presence in the subsidiary histone from calves' thymocytes is

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

evident from the chromatogram; no corresponding spot has, however, been observed in the chromatograms of the main histones. It is just possible that it has been masked by the adjacent spot of the leucines which are present in high amount, although this seems doubtful. According to Allgén (1950) however, it is present in thymus histone prepared from the whole gland of the calf. This may be due to a contaminant from the cytoplasm or to the presence of a subsidiary histone. In a communication which followed the publication (Stedman & Stedman 1950*a*) of a summary of a few of the results now described, Hamer (1951) has recorded a complete amino-acid analysis of thymus histone from the calf and stated that no cysteine or cystine could be detected in it. No mention is made of methionine.

The chromatograms of clupeine and salmine are of special interest, for these two protamins have, in the past, formed the subject of numerous investigations. This has doubtless been due to their low molecular weights, which cause them to differ from other proteins in forming true solutions in water. They were at first thought to be identical, but a definite difference between them was apparently detected when Kossel and Dakin (see Kossel 1928) found that alanine was present in clupeine but absent from salmine. According to this older work, clupeine was composed of arginine, alanine, serine, valine and proline, while the amino-acids present in salmine were arginine, serine, valine and proline. The subsequent work on clupeine has been marred either by the use of impure material or by the partial decomposition of the base by fractionating it in hot solution (see p. 587), while work on salmine has usually been made on commercial preparations, the history and method of preparation of which cannot be known. Using such material, Block & Bolling (1945*b*), in their amino-acid analyses, accounted for 100 % of the nitrogen of salmine. In this work they detected the presence for the first time of alanine and small amounts of isoleucine, results which were later confirmed by Tristam (1947) who also detected, again for the first time, the presence of glycine. Tristam's analyses accounted for 99·3 % of the total nitrogen. The discovery of the presence of alanine in salmine removes the principal proof which has hitherto existed of the non-identity of salmine and clupeine. While the arginine contents of the two proteins are, as shown in table 5, different, they are nevertheless so close to one another as to fall almost within the limits of experimental error. In order to confirm the difference between them we have submitted the preparations 111X1 of salmine and 11V*b*1 of clupeine to chromatographic analysis with the following results. Salmine was found to contain glutamic acid, serine, glycine, alanine, arginine, proline and valine. It should be noted that our results differ from Tristam's in that glutamic acid is present and isoleucine absent. The amount of glutamic acid indicated in the chromatogram is small relatively to most of the monoamino-acids, but so also is that of alanine. If, therefore, glutamic acid is to be attributed to an impurity, alanine must probably be placed in the same category. There is, however, no reason to suspect the purity of the salmine. The amino-acid composition of clupeine differs from that of salmine. It contains: glycine, serine, alanine, threonine, arginine, proline, valine and isoleucine (or leucine). The amounts of glycine and isoleucine present were, however, small.

The small fraction X2, obtained from the mother liquors from the purification of salmine, contained the amino-acids: aspartic and glutamic acids, glycine, serine, alanine,

lysine, arginine, proline, valine and isoleucine (or leucine). This was almost certainly contaminated with a small quantity of salmine, but the presence of lysine and other amino-acids which are not present in salmine suggests that it may contain a protein of the nature of a subsidiary histone. The presence of this protein might well account for the isoleucine found in Block & Bolling's and Tristam's preparations of salmine.

Discussion

There remains for discussion the question of the significance of the cell specificity, demonstrated in the course of this investigation, of the basic proteins present in cell nuclei. Before considering this problem, however, it should be pointed out that these basic proteins constitute quantitatively major components of cell nuclei, for they represent from 20 to 30 % of the dry weight of the various nuclei examined. As such they must in part be responsible for the formation of one or other of the chief structures of the nucleus. It has been shown elsewhere (Stedman & Stedman 1947*a*, *b*) that, in combination with nucleic acid, they do, in fact, form the nuclear sap in the resting and prophase, and the spindle in the metaphase, nucleus. For the exercise of such structural functions, which cannot of course be wholly divorced from physiological ones, cell specificity would presumably be inessential. In order to gain some insight into the meaning of the specificity it is therefore necessary to attempt to visualize the more purely physiological functions of the basic proteins. Unfortunately, the mechanism by which the nucleus itself exerts its functions, whatever these may be, is little understood. Nevertheless, it is a principle of cytogenetics that each nucleus of an organism contains at least one set of the chromosomes, and hence all of the genes, of that organism. It is, therefore, presumably through the activity of these hypothetical units that the function of the nucleus must be exercised. There is, however, one difficulty inherent in this view. If all the genes are active in every nucleus of an organism, any one nucleus would be physiologically identical with another. The action of individual genes would not then be limited, as the evidence indicates that they are, to certain types of cells but would extend to every cell in the organism. Moreover, it would be impossible on the chromosome theory of heredity to account for cell differentiation, for this process implies that different groups of genes are active in different types of cells. The unequivocal demonstration in this work of the cell specificity of histones and protamins in at least some cells shows that although, as would be expected from cytological theory, cell nuclei do resemble one another closely in general chemical composition, there are nevertheless differences in the composition of the basic proteins in the nuclei of cells of different types. This must be associated with differences in the physiological properties of these nuclei and, if it is correct that the functions of a nucleus are due to the genes which it contains, it follows that the action of the basic proteins in modifying the properties of the nucleus must be exercised through an action on the genes. We therefore advance the hypothesis that the basic proteins of cell nuclei are gene inhibitors, each histone or protamin being capable of suppressing the activities of specific groups of genes. Such an hypothesis will, we believe, not only serve to explain the different properties of cell nuclei, but will also give some indication of the mechanism by which the nucleus effectively participates in the process of cell differentiation.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

We have to acknowledge with thanks Grants from the Royal Society and the Medical Research Council which have, in different ways, assisted this investigation. We should also like to thank Dr Helen Russell of the Christie Hospital and Holt Radium Institute for providing us with the human tissue used; Dr A. W. Greenwood and Miss J. Peace of the Poultry Research Centre for the supply of chickens; and Mr G. J. Dutton of this Department for procuring the salmon livers.

Description of Plate 20

Figure 1. Wet smear of finished preparation of salmon sperm heads. Stained methylene blue. (Magn. × *ca.* 200).

Figure 2. Salmon sperm heads after treatment with 70% alcohol. Stained methylene blue. (Magn. × *ca.* 700).

Figure 3. Paper chromatogram of amino-acids from the histone isolated from the sperm heads of the cod. (1) aspartic acid; (2) glutamic acid; (3) glycine; (4) serine; (5) alanine; (6) threonine; (7) lysine; (8) arginine; (9) histidine; (10) proline; (11) valine; (12) the leucines with, possibly, methionine; (13) phenylalanine; (14) tyrosine.

Figure 4. Paper chromatogram of amino-acids from the subsidiary histone isolated from nuclei from the thymus gland of the calf. (1) aspartic acid; (2) glutamic acid; (3) glycine; (4) serine; (5) alanine; (6) threonine; (7) lysine; (8) arginine; (10) proline; (11) valine; (12) leucine or isoleucine; (15) methionine.

References

Ackermann, D. 1904 *Hoppe-Seyl. Z.* **43**, 299.
Allgén, L. 1950 *Acta Physiol. Scand.* **22**, Suppl. 76.
Block, R. J. & Bolling, D. 1945*a* *The amino-acid composition of proteins and foods*. Springfield: Thomas.
Block, R. J. & Bolling, D. 1945*b* *Arch. Biochem.* **6**, 419.
Calvet, F., Siegel, B. M. & Stern, K. G. 1948 *Nature, Lond.*, **162**, 305.
Consden, R., Gordon, A. H. & Martin, A. J. P. 1944. *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H. & Martin, A. J. P. 1947 *Biochem. J.* **41**, 590.
Di Stefano, H. S. 1948 *Chromosoma*, **3**, 282.
Ely, J. O. & Ross, M. H. 1949 *Anat. Rec.* **104**, 103.
Felix, K. & Rauch, H. 1931 *Hoppe-Seyl. Z.* **200**, 27.
Hamer, D. 1951 *Nature, Lond.*, **167**, 40.
Kossel, A. 1884 *Hoppe-Seyl. Z.* **8**, 511.
Kossel, A. 1928 *The protamines and histones*. London: Longmans.
Lamb, W. G. P. 1949 *Nature, Lond.*, **164**, 109.
Lilienfeld, L. 1894 *Hoppe-Seyl. Z.* **18**, 473.
Macpherson, H. T. 1946 *Biochem. J.* **40**, 470.
Miescher, F. 1897 *Die histochemischen und physiologischen Arbeiten* **1**, **2**. Leipzig: Vogel.
Mirsky, A. E. & Pollister, A. W. 1942 *Proc. Nat. Acad. Sci., Wash.*, **28**, 344.
Mirsky, A. E. & Pollister, A. W. 1943 *Biol. Symp.* **10**, 247.
Mirsky, A. E. & Pollister, A. W. 1947 *J. Gen. Physiol.* **30**, 117.
Mirsky, A. E. & Ris, H. 1948 *J. Gen. Physiol.* **31**, 1, 7.
Plimmer, R. H. A. & Rosedale, J. L. 1925 *Biochem. J.* **19**, 1020.
Pollister, A. W. & Ris, H. 1947 *Cold Spr. Harb. Symp. Quant. Biol.* **12**, 147.
Pollister, A. W. & Leuchtenberger, C. 1949 *Proc. Nat. Acad. Sci., Wash.*, **35**, 66.
Ris, H. & Mirsky, A. E. 1949 *J. Gen. Physiol.* **32**, 489.
Ris, H. & Mirsky, A. E. 1950 *J. Gen. Physiol.* **33**, 125.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

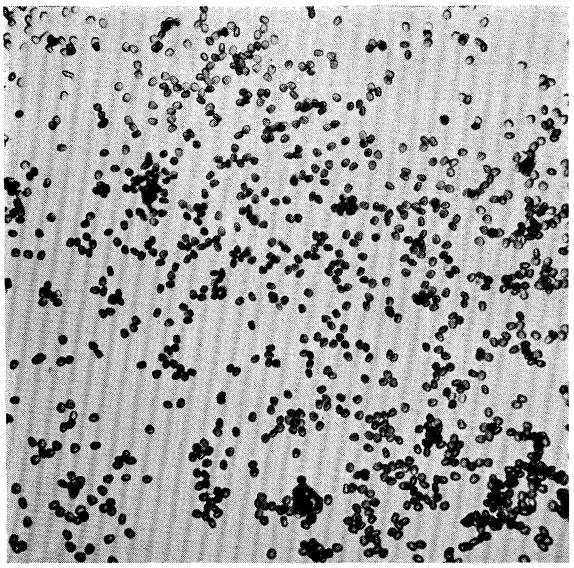

FIGURE 1

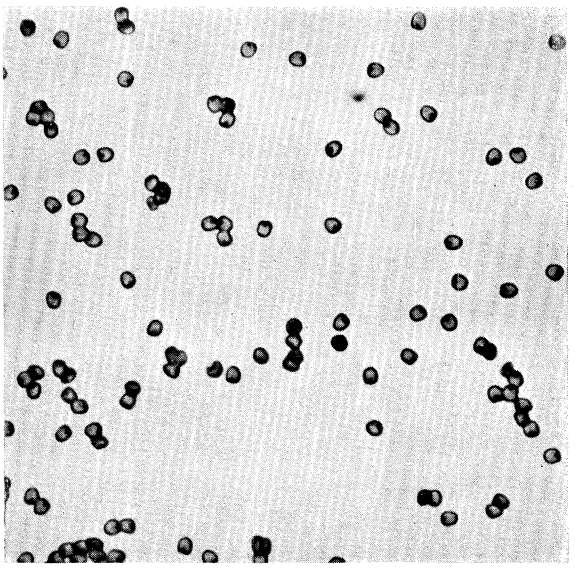

FIGURE 2

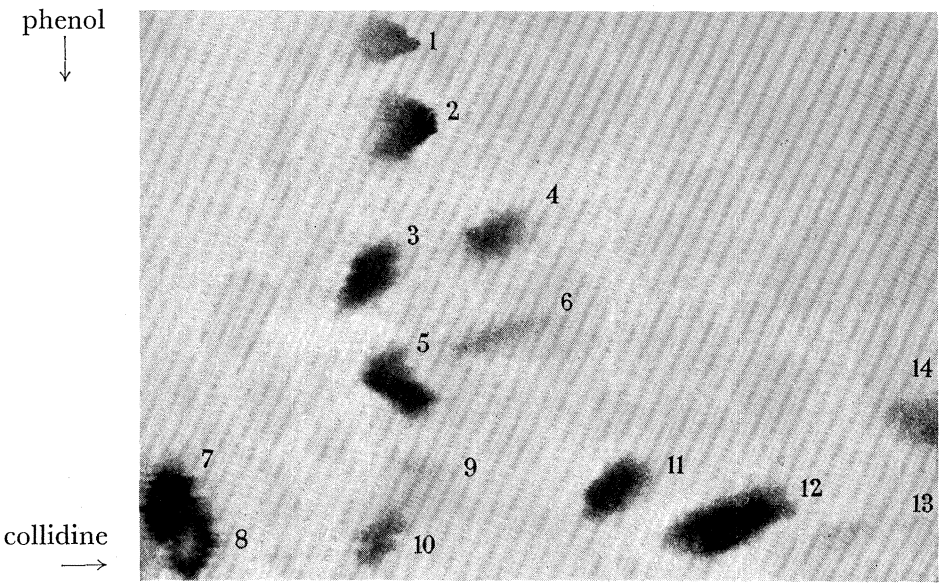

FIGURE 3

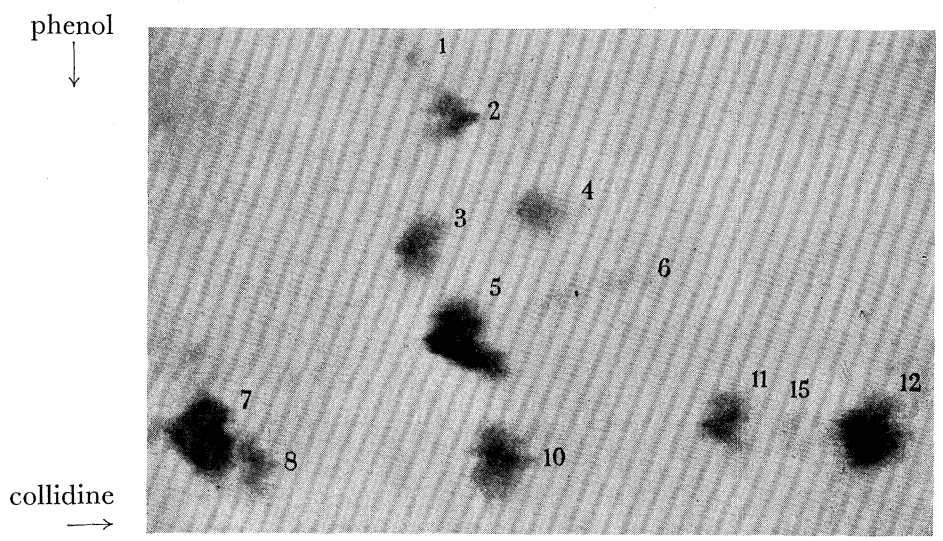

FIGURE 4

(*Facing p.* 594)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Schmitt, F. O. 1944 *Adv. Protein Chem.* **1**, 28.
Stedman, E. 1944 *Edinb. Med. J.* **51**, 353.
Stedman, E. & Stedman, E. 1943 *Nature, Lond.*, **152**, 267.
Stedman, E. & Stedman, E. 1944 *Biochem. J.* **38**, xxvi.
Stedman, E. & Stedman, E. 1947*a* *Symp. Soc. Exp. Biol.* **1**, 232.
Stedman, E. & Stedman, E. 1947*b* *Cold Spr. Harb. Symp. Quant. Biol.* **12**, 224.
Stedman, E. & Stedman, E. 1948 *Biochem. J.* **43**, xxiii.
Stedman, E. & Stedman, E. 1950*a* *Nature, Lond.*, **166**, 780.
Stedman, E. & Stedman, E. 1950*b* *Biochem. J.* **47**, 508.
Steudel, H. & Peiser, E. 1922 *Hoppe-Seyl. Z.* **122**, 298.
Tristam, G. R. 1947 *Nature, Lond.*, **160**, 637.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

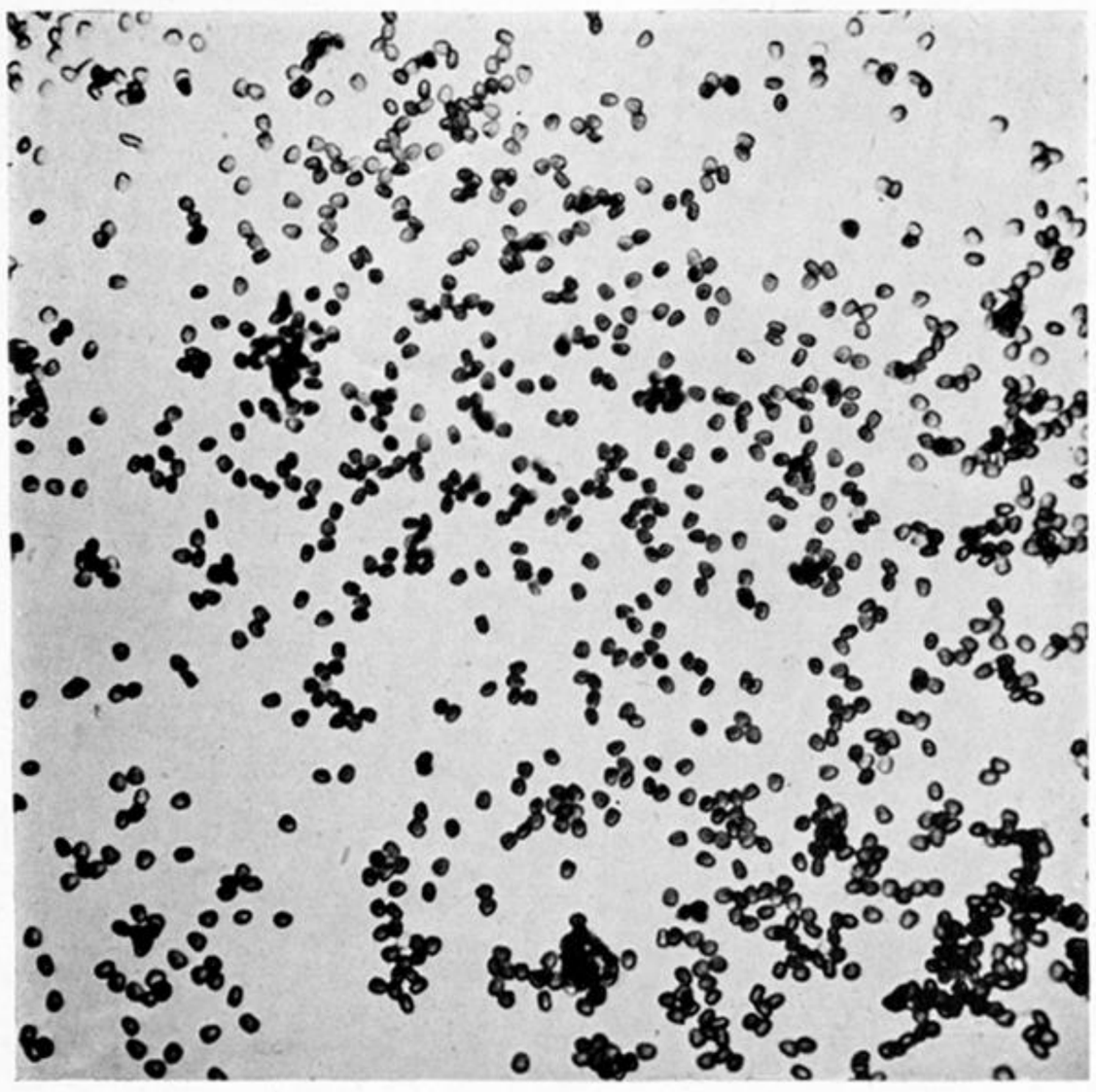

FIGURE 1

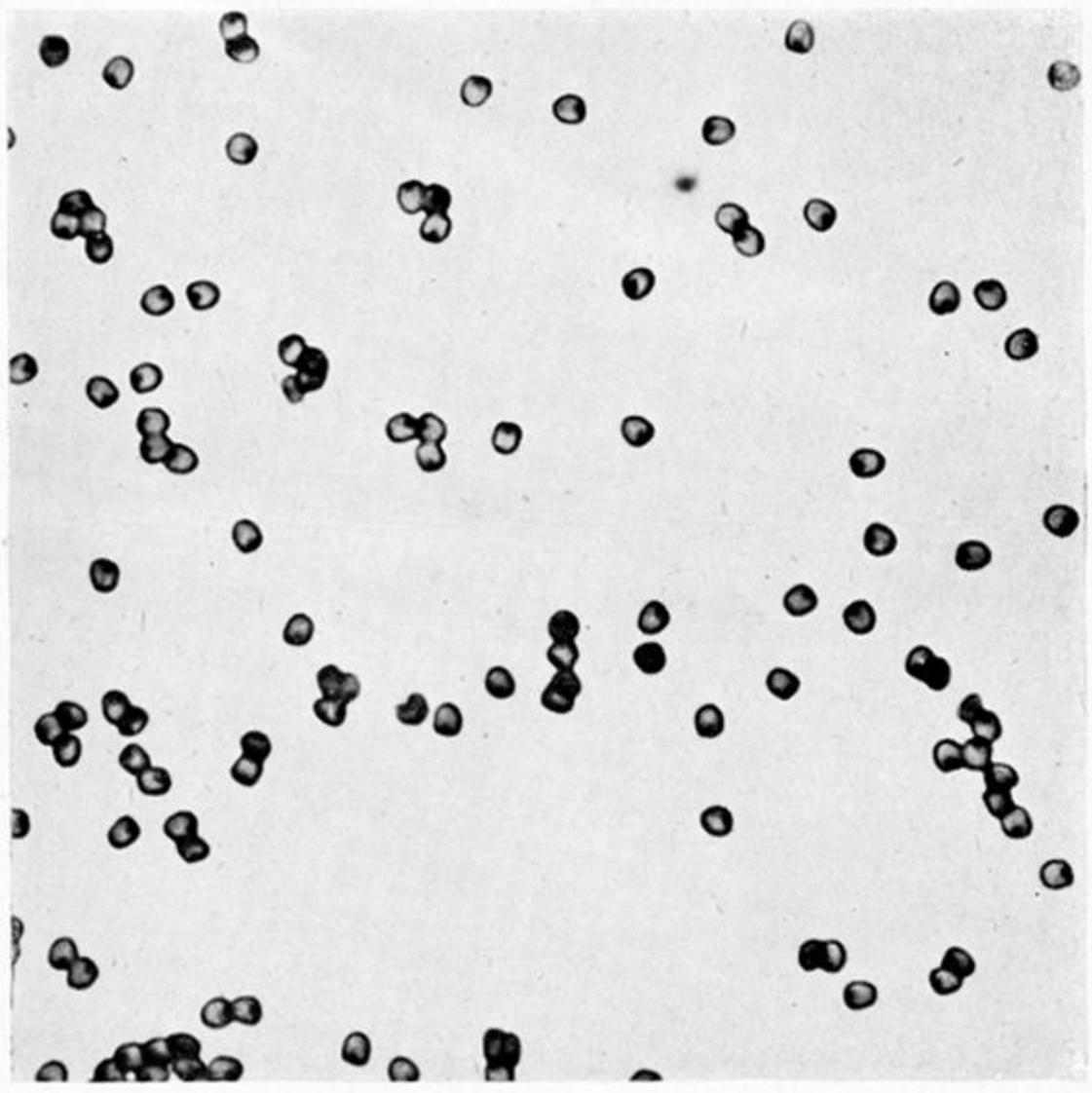

FIGURE 2

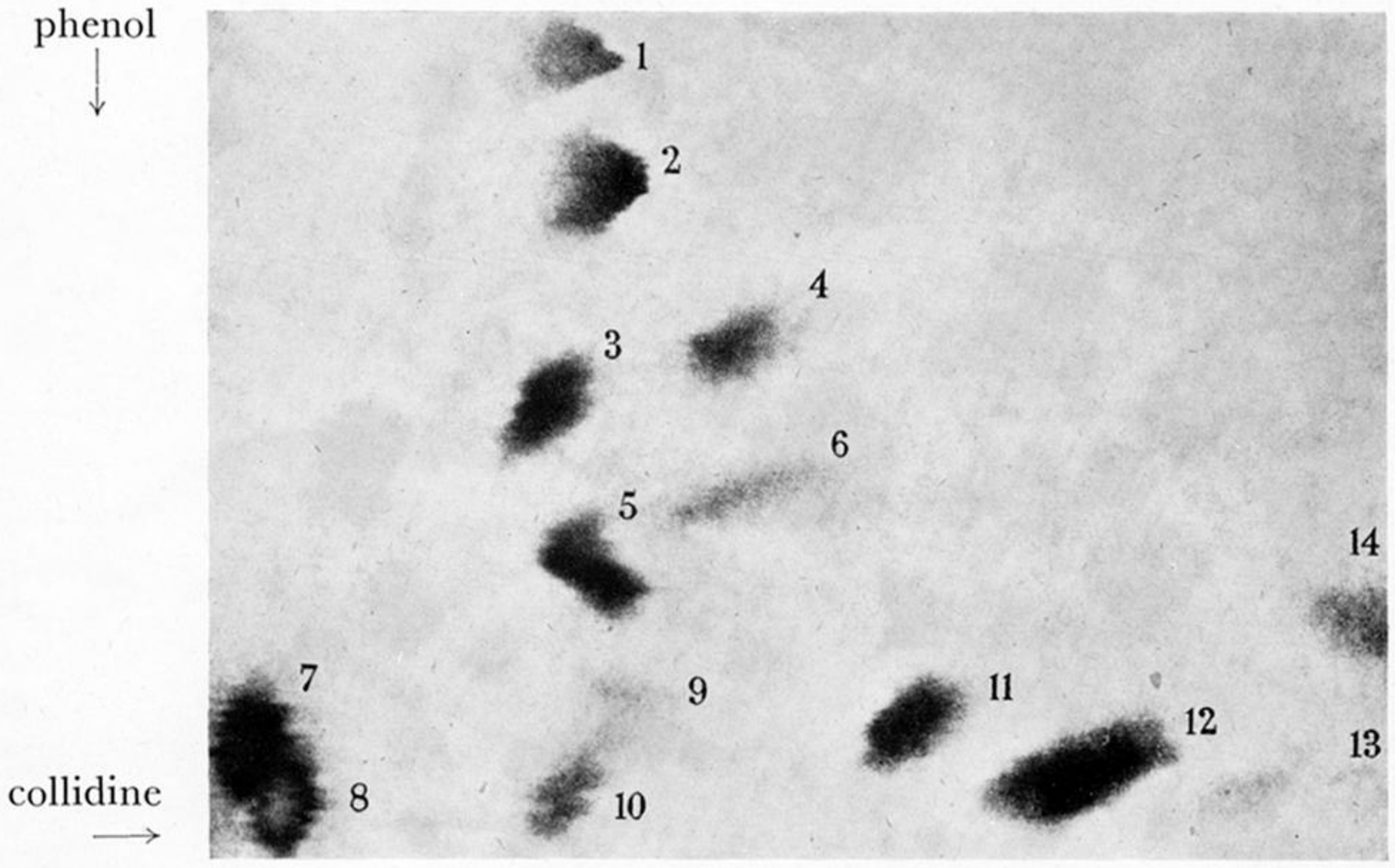

FIGURE 3

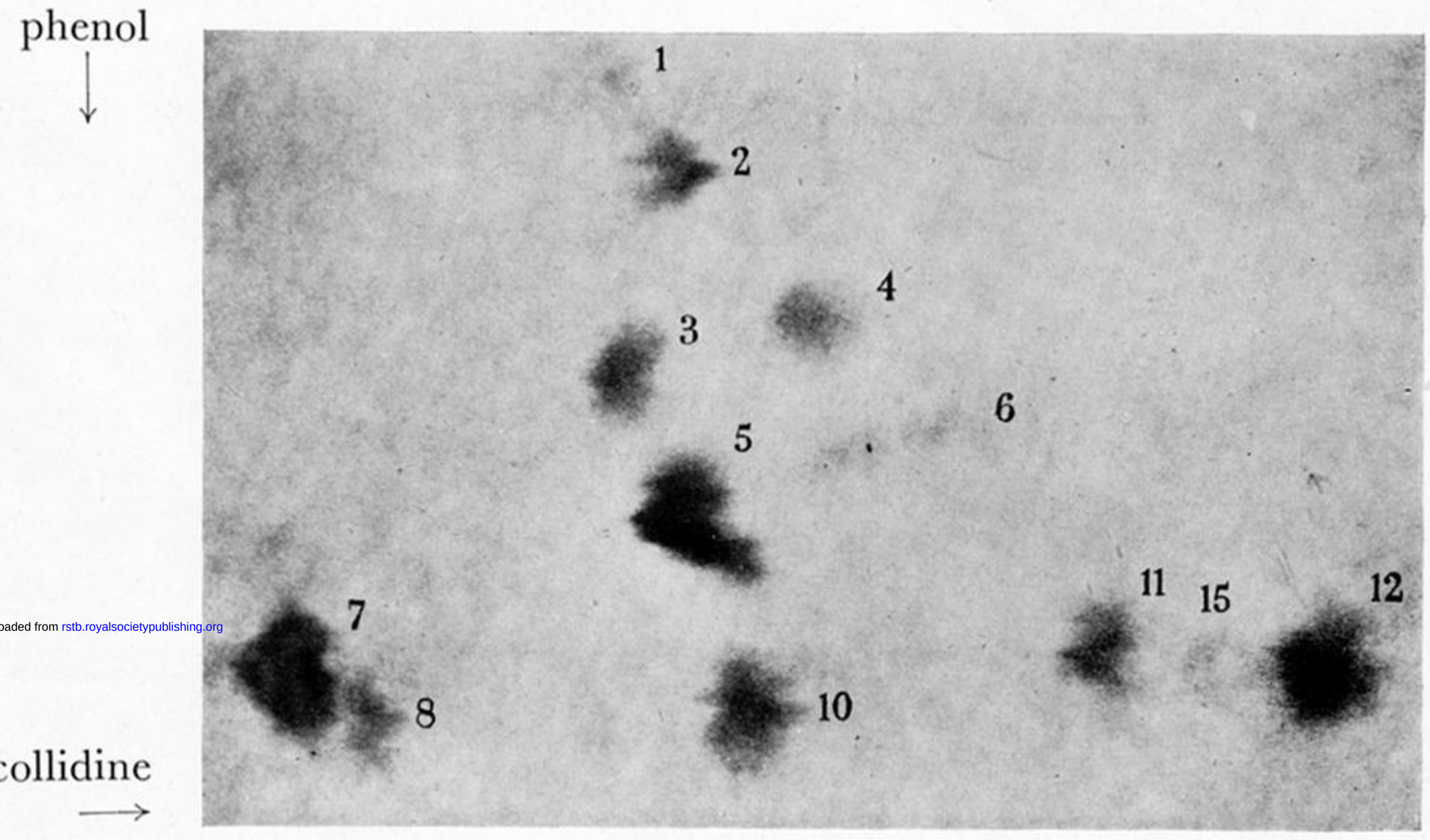

FIGURE 4

DESCRIPTION OF PLATE 20

FIGURE 1. Wet smear of finished preparation of salmon sperm heads. Stained methylene blue. (Magn. × *ca.* 200).

FIGURE 2. Salmon sperm heads after treatment with 70% alcohol. Stained methylene blue. (Magn. × *ca.* 700).

FIGURE 3. Paper chromatogram of amino-acids from the histone isolated from the sperm heads of the cod. (1) aspartic acid; (2) glutamic acid; (3) glycine; (4) serine; (5) alanine; (6) threonine; (7) lysine; (8) arginine; (9) histidine; (10) proline; (11) valine; (12) the leucines with, possibly, methionine; (13) phenylalanine; (14) tyrosine.

FIGURE 4. Paper chromatogram of amino-acids from the subsidiary histone isolated from nuclei from the thymus gland of the calf. (1) aspartic acid; (2) glutamic acid; (3) glycine; (4) serine; (5) alanine; (6) threonine; (7) lysine; (8) arginine; (10) proline; (11) valine; (12) leucine or isoleucine; (15) methionine.