ADVANCES IN PROTEIN CHEMISTRY

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PREFACE

The first chapter in this volume of the *Advances* is by the late Professor Kurt Felix. His careful investigations of the structure and general biological significance of the protamines have continued without interruption since his early collaboration with A. Kossel, who first demonstrated that these substances were composed of amino acids and were, consequently, proteins. The chapter includes a thorough compilation of the historical development of this branch of protein chemistry, together with an evaluation of its present chemical and biological status. The advanced state of knowledge of the protamines is of particular importance today because of the increasing interest in the structural and functional interrelationships that appear to exist between these substances and the DNA molecules with which they are associated in the nuclei of cells.

In the second chapter of the volume, Donald Kupke presents a critical and practical discussion of the use of osmotic pressure determination as a tool in the study of proteins and other macromolecules. Osmotic pressure measurements have not commonly been employed by protein chemists in recent years due, perhaps, to the complexities of the usual apparatus and to the popularity of other methods for the determination of molecular weights. Kupke describes several relatively simple but powerful modifications of the technique that would make valuable additions to the usual physicochemical procedures used in laboratories concerned with protein chemistry and which yield information not easily available by other procedures.

The third article, by Waterlow, Cravioto, and Stephen, deals with protein malnutrition in man. This major international problem is an extremely complex one since its manifestations, diagnosis, and treatment are dependent on numerous other nutritional deficiencies and metabolic disturbances and involve the difficult problems of the availability and suitability of foodstuffs. The present review is a detailed and illuminating discussion of the many scientific and sociological factors that are associated with the disease. Because of its potential value to those working in the field, this article is being reprinted for distribution through UNICEF and other organizations concerned with protein malnutrition, and we would like to thank the authors and the staff of Academic Press for their efforts in making this possible.

In the fourth article of the volume, Christensen considers the problem of the transport of substances across cell membranes. His thorough and critical review of the subject is of general applicability to all aspects of

biological transport. The article belongs, quite reasonably, in the *Advances* because so much of the work in this field has centered around the transport of amino acids. Christensen takes, as his major theme, the chemical nature of the macromolecules that form the cell wall and develops a strong case for the importance of specific interacting groups on such molecules as major determinants of selectivity in transport. His presentation emphasizes the probable value of applying modern techniques of protein chemistry to cell wall components that can be shown to undergo unique association with molecules in transport.

The last chapter, by Czok and Bücher, is a comprehensive review of the so-called myogen fraction of muscle. This fraction, which is essentially the juice that can be pressed from muscle fibers mechanically, contains most of the enzymatically active proteins of the tissue. The authors describe techniques for the isolation and crystallization of these enzymes and present a particularly valuable treatment of the process of controlled salt fractionation as employed in their laboratory. The reactions catalyzed by each of the individual enzyme components are considered in detail, and the distribution of enzymes in different muscles is summarized in a useful section on the comparative biochemistry of the myogen fraction.

We would like to express our appreciation to the staff of Academic Press for their dependable and expert cooperation in the preparation of this volume of *Advances*. Our thanks are also due Dr. F. Netter and Mrs. Lisa Barnett for their efforts in translating the articles by Drs. Czok and Bücher and by Professor Felix, and to Dr. A. Goppold-Krekels for her kind assistance in expediting the handling of proofs on the latter article.

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December, 1960

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PROTAMINES

By KURT FELIX

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I. Historical

The first protamine was isolated by Miescher (1874) from salmon sperm. He extracted the fat-free sperm with dilute (1–2 %) hydrochloric acid, neutralized the excess acid, and treated the solution with platinum chloride. All the protamine precipitated as the double salt of platinum and was free of sulfur and phosphorus. According to Piccard (1874) it still contained traces of guanine and hypoxanthine. The precipitate was insoluble in water, but soluble in an excess of hydrochloric acid. A similar platinum salt was obtained by him from the acid extract of carp sperm. While he

considered salmon sperm protamine to be a simple nitrogenous base, he observed that the corresponding material from carp sperm gave reactions for protein.

The protamine of salmon, later named salmine, which can be extracted with hydrochloric acid and precipitated with platinum chloride, corresponds to about 26.8 % of the dried sperm (Miescher, 1874).

About 20 years later, Kossel (1896) showed that the protamines are made up of amino acids and are therefore proteins. He repeated Miescher's experiments and obtained the same material from salmon sperm. However, since he was unable to obtain sufficient amounts of starting material for further investigations, he worked primarily on the sperm of sturgeon (*Acipenser sturio*) which were obtainable in adequate amounts from the Baltic Sea. He also simplified the preparation to some extent. The mature testes were minced, shaken with water, strained through a cloth, and the turbid liquid was acidified with a few drops of acetic acid, thereby conglomerating particulate elements so that they could be filtered off easily. The residue on the filter was extracted several times with alcohol and ether and then dried. The dried material was shaken with 5 volumes of 1 % sulfuric acid and any insoluble material was filtered off. The extraction was repeated three times with the same amount of sulfuric acid. The protamine sulfate was precipitated from the filtrate with a threefold excess of alcohol. The sulfate is very soluble in water and may be washed further by repeatedly redissolving it and reprecipitating it with alcohol. The salmine sulfate from salmon sperm was precipitated as the picrate from aqueous solution by sodium picrate and then reconverted into the sulfate. The protamine from sturgeon sperm (sturine) was quite similar to salmine, but differed from it in composition and in some of its reactions (Kossel, 1896). Among the hydrolysis products of sturine Kossel discovered histidine (Kossel, 1898). Along with his co-workers and students, he continued to isolate further protamines from the sperms of several species of fish.

II. Preparation of Protamines

The preparation of protamines continues, in general, to follow the method outlined by Miescher and Kossel, which is based on the fact that the protamines occur as salts of nucleic acids in the nuclei of fish spermatozoa. The nucleic acid salts can be dissociated by treatment with dilute sulfuric acid (1 ml of concentrated H_2SO_4 made up to 100 ml with water) or with 1 % hydrochloric acid. In view of the possibility that, during extraction, the nucleic acid may be partially broken down and some of its bases may go into solution, the protamine must be precipitated and washed. Although the sulfate of the protamine can be precipitated with 3 volumes of alcohol, it is not insoluble enough to be washed thoroughly. To precipitate the chloride, acetone is needed.

The combination with heavy metals has not proven successful, since the metals have to be removed as their sulfides and the precipitates occlude considerable amounts of nitrogenous material. Besides platinum chloride, mercuric nitrate in nitric acid solution can be used for precipitation of protamines, or alkaloid precipitants may be used. The most suitable products are the picrates since they are highly insoluble and can be washed thoroughly, even with 0.1 *N* sodium hydroxide which will remove any purine picrate that may also have precipitated. Precipitation is carried out, not with alcoholic picric acid, but with aqueous sodium picrate which reacts almost exclusively with the strongly basic protamines and with very few other bases. The picrates are taken up in sulfuric acid and the picric acid is removed by extraction with ether or toluene. The sulfates are then reprecipitated with alcohol.

The best starting material for the preparation of protamines is freshly drawn milt from fish that are ready to spawn, since it contains only one type of cells, the spermatozoa. If sufficient milt cannot be obtained, the mature testes are used as described above in the historical introduction. Occasionally these contain other cells besides the sperm, such as earlier stages of sperm and connective tissue cells; the more immature the testes, the less homogeneous the cellular material. The protamines obtained therefrom may still be contaminated with other proteins or their decomposition products. It is important to disperse the minced testes in 4 or 5 volumes of water and to shake the mixture vigorously. The material is then strained through muslin or a wire sieve. The extract is acidified with acetic acid until it just gives a blue color with Congo red paper. The precipitate is collected on fluted filter paper, freed from fatty material, and dried as described above. To prepare the protamine from the dried material, Kossel (1929) describes two procedures. The first of these, based on extraction with acid, is preferred by most authors. The original directions are as follows.

A. Procedures of Kossel

(1) Approximately 100 gm of the powdery material are shaken with 500 ml of 1 % sulfuric acid for ½ hr and then filtered. The residue on the filter is extracted several times, until an aliquot of the sulfuric acid extract gives no visible precipitate with alcohol. The extraction should take no longer than 1 day; if the sperm material is left in contact with the sulfuric acid for a longer period, some decomposition of nucleic acids and formation of interfering substances will result. The protamine sulfate is precipitated from the sulfuric acid extract by addition of 3 volumes of alcohol and the supernatant is decanted or siphoned off. The precipitate is then dissolved in a little hot water and the alcohol precipitation is repeated. The total amount of precipitate obtained from 100 gm of air-dried sperm mate-

rial is then dissolved in about 1 or 2 liters of hot water. On cooling, a small amount of the sulfate separates out as a yellow or brownish oil. This most insoluble fraction of the protamine sulfate is separated from the supernatant liquid and the latter evaporated to a small volume. The bulk of the oil is then obtained by means of a separatory funnel, the middle fraction of the oil being the most pure.

The protamine sulfate, after being dissolved in warm water, is purified further by precipitation with sodium picrate, followed by the removal of picric acid by shaking the well-washed precipitate with toluene in the presence of an excess of sulfuric acid, and then protamine sulfate is precipitated from the sulfuric acid solution with alcohol. The alcohol precipitation is then repeated. The consistency of the precipitate is dependent upon the degree of acidity of the solution; if the amount of acid is inadequate, a turbid solution is obtained. In that case, flocculation can be effected by careful, dropwise addition of sulfuric acid. The formation of a sticky precipitate indicates that too much acid is present and solution in water and precipitation with alcohol must be repeated. The protamine sulfate should come down as a powdery, pure white precipitate. Then this is washed with alcohol and ether and dried in a desiccator. The yield, for mature herring testes, should be 15–20 % of the air-dried sperm material.

When larger quantities are to be prepared, the amount of alcohol may be reduced by evaporating the sulfuric acid extracts to a smaller volume. However, it should be noted that the extract contains small amounts of other tissue constituents which are removed by the alcohol precipitation described above, but which, during evaporation, will partially decompose and adhere to the preparation.

Then the sulfuric acid extracts are treated as follows: the combined extracts are neutralized with barium hydroxide and evaporated in a water bath. The extracts are reduced to a volume suitable for the separation of the oil, which is then purified with picrate as described above.

This method of preparation may be used for most protamines. With the protamines of carp sperm, however, the oily separation of the sulfate does not occur.

(2) In the second procedure, the dried sperm material is treated with cupric chloride solution. The protamines pass into solution, while the nucleic acids form insoluble copper salts. The protamines are precipitated from the filtrate with sodium picrate. This method was developed by Nelson-Gerhardt (1919) of Kossel's Institute, making use of data by Schmiedeberg (1899) and Malenük (1908). Since the method is still used by some authors, the details of the procedure are described below.

One hundred grams of dried sperm material, prepared as described above, are incubated in an oven with a solution of 100 gm of cupric chloride in 1

liter of water. Incubation is continued for 3 days, with occasional shaking. Then the supernatant liquid is filtered off with suction. The residue is resuspended in water three times and filtered with suction. Washing of the residue is continued until an aliquot of the filtrate, when tested with concentrated sodium picrate solution, does not show a precipitate.

Concentrated sodium picrate solution is added, with stirring, to the combined filtrates and washings until the precipitate sticks together and settles out quickly. The precipitate is filtered off, washed with a little water to which some sodium picrate has been added, and dissolved while still wet, with slight heating, in a mixture of 1 volume of acetone and 3 volumes of water. If possible, the solution is filtered and the filtrate treated with half the volume of alcohol. Sulfuric acid (20% v/v) is then added dropwise, with stirring, until no further precipitate forms. An excess of sulfuric acid will render the precipitate smeary and soluble and therefore must be avoided. The supernatant liquid is decanted through a filter and the residue treated with absolute alcohol, thus being made hard and crumbly. The precipitate is decanted several times with alcohol and finally with ether, filtered off, and washed with ether. Then the ether is removed in a desiccator. Further purification of the preparation may be accomplished, making use of its resistance to the action of pepsin. An aqueous solution of 10 gm of protamine sulfate is digested for 24 hr at 37°C in about 250 ml of water to which 0.1 gm of commercial pepsin and 0.5 gm of HCl have been added. The digestion mixture is neutralized with sodium carbonate and the protamine picrate is precipitated, as described above, and converted to the sulfate, which is then made to solidify, and is redissolved as in procedure (1).

1. Conversion of Protamine Picrate to Protamine Ester Hydrochloride

From picrate suspended in dilute sulfuric acid, picric acid can be removed only by repeated extraction with toluene or ether. This removal may be accomplished faster by suspending the dry picrate in 100 volumes of anhydrous 0.2 *N* hydrochloric acid in methyl alcohol and letting it stand for 24 hr, with occasional stirring. The residue is filtered off with suction, dried, shaken with fresh methyl alcoholic hydrochloric acid (about one-fourth the amount used before), allowed to stand 24 hr, filtered as before and the residue discarded. The combined filtrates are treated with 3 volumes of anhydrous ether, allowed to stand overnight in the cold room, and the protamine methyl ester hydrochloride precipitate is filtered off with suction. The crude product is redissolved in the 0.2 *N* methyl alcoholic hydrochloric acid, reprecipitated with 3 volumes of anhydrous ether, and finally dried with phosphorus pentoxide under vacuum (Felix and Dirr, 1929; Felix and Mager, 1937c; Rauen *et al.*, 1952).

2. Purification of Protamine Sulfate by Refractionation (Umscheiden)

Waldschmidt-Leitz and co-workers employ a "refractionation" procedure for the purification of clupeine sulfate. The alcohol and ether dried testes are extracted with cupric chloride (as described in Section II, *A*), precipitated with sodium picrate, the picrate converted to the sulfate, and the latter precipitated with acetone. A saturated solution of the sulfate at 30–40°C (about 7 %) is prepared and this is cooled to 0°C, with the resultant separation of the clupeine sulfate as an oily liquid. This separation is repeated many times over a period of several weeks. (Rauen *et al.*, 1952; Waldschmidt-Leitz *et al.*, 1931; Waldschmidt-Leitz and Voh, 1954; Waldschmidt-Leitz and Mindemann, 1956; Waldschmidt-Leitz and Gudernatsch, 1957.)

B. Procedure of Ando and Yamasaki

Ando *et al.* (1957c) and Yamasaki (1958) recommend the following modification of Kossel's procedure.

Frozen herring milt (410 gm) is finely powdered and stirred into 1.6 liters of 0.05 *M* sodium citrate, pH 7. Then it is strained through cheesecloth and the filtrate is centrifuged for 10 min at 4000 rpm. The pellet is suspended again in 1.6 liters of the citrate solution, stirred for 30 min, and then centrifuged at a lower speed (1300 rpm) for 2 min. The brown sediment (tissue debris) is discarded and the supernatant is centrifuged for 5 min at 4000 rpm.

The wet sperm heads are stirred vigorously in 4 liters of 2 *M* NaCl containing 0.05 *M* sodium citrate. This dissolves the nucleoclupeine. While stirring, 400 ml of a saturated $CuSO_4$ solution are added to the mixture, thus precipitating the nucleic acids as their copper salts. Stirring is continued for additional 30 min and the mixture is allowed to stand overnight in the cold room. The copper salts of the nucleic acids are then filtered off and washed with water. From the combined filtrates and washings (6 liters, the clupeine is precipitated as its picrate by treatment with 0.125 *M* sodium picrate (400 ml).

Ten grams of the picrate are dissolved at room temperature in 100 ml of aqueous acetone solution (67 % w/v) and the insoluble material is filtered off. At this point, fractionation can be accomplished by cooling, the less soluble picrate coming down as an oil. Both picrates are converted to the sulfates by addition of 2 *N* sulfuric acid, and the sulfates are precipitated with alcohol. Then they are dissolved in water and reprecipitated with alcohol. By means of an anion exchanger (Amberlite IRA-400, Cl form), they may be converted to the hydrochlorides.

C. Procedure of Block et al.

Block and Bolling recommend a different procedure. From a suspension of spermatozoa, the heads are precipitated with 1% citric acid, extracted with 0.2% hydrochloric acid, and the extract is heated to the boiling point. The pH is adjusted to 8 with ammonia and the precipitate is filtered off. The protamine is precipitated from the filtrate with the optimal amount of 33% metaphosphoric acid, and the protamine metaphosphate is converted to the sulfate with 1 *N* sulfuric acid. The sulfate is then precipitated in the usual manner with alcohol or acetone and dried. This method, while perhaps producing a larger yield, might also cause some hydrolysis (Block *et al.*, 1949; Rauen *et al.*, 1953).

D. Procedure of Felix et al.

Recent procedures use purified spermatozoa nuclei as the starting material. From these, nucleoprotamines are prepared, and from them, the protamines are obtained.

1. Preparation of Fish Sperm Nuclei

a. Principle. On treating the filtrate from an aqueous suspension of minced testes with acetic acid, the sperm heads are precipitated as nuclei together with some cytoplasmic material. A purer and more homogeneous preparation is obtained by starting with the nuclei after complete removal of any cytoplasm. This is done by placing the spermatozoa in distilled water. Within a few minutes, the cytoplasm in the head and tail begins to swell. The fibrils of the latter form loops, while the nucleus itself remains unaltered. Then the nuclei can be separated from the cytoplasm and tails by homogenizing the suspension briefly (5 min) and centrifuging. The fibrils and cytoplasm remain in the supernatant, which gives a reaction for tyrosine, tryptophan, cystine, and ribonucleic acid. The nuclei are in the sediment. They are washed in the centrifuge with distilled water until the above reactions are negative, indicating that the cytoplasm has been completely removed (Felix *et al.*, 1951b). The above amino acids, as well as ribonucleic acid, are missing from the nuclei of trout and herring sperm. This method of preparation does not impair the biological potency of the nuclei; it has been possible, in a few cases, to use them to fertilize the corresponding fish eggs and to promote the development of normal fish (Felix *et al.*, 1952b) (see Figs.1–5).

When the nuclei are precipitated with acetic acid, they are gray rather than pure white and sometimes give a reaction for tyrosine (Steudel, 1911; Felix *et al.*, 1951b).

FIG. 1. Trout sperm in Ringer solution. Osmium fixation. Magnification: × 11,000. Electron micrographed by Drs. W. Lippert and O. Hug of the Max Planck Institute for Biophysics, Frankfurt/Main.

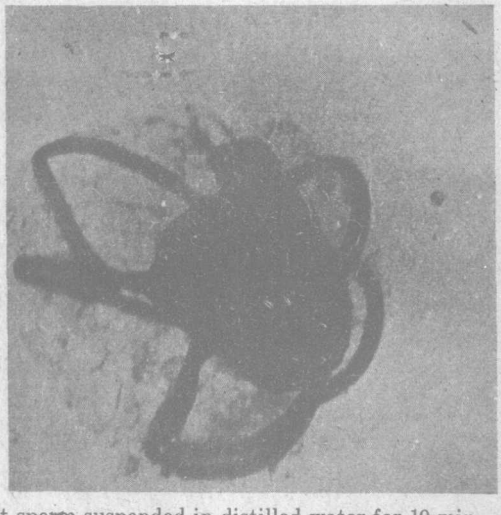

FIG. 2. Trout sperm suspended in distilled water for 10 min. Osmium fixation. Magnification: × 10,000. Electron micrograph by Drs. W. Lippert and O. Hug of the Max Planck Institute for Biophysics, Frankfurt/Main.

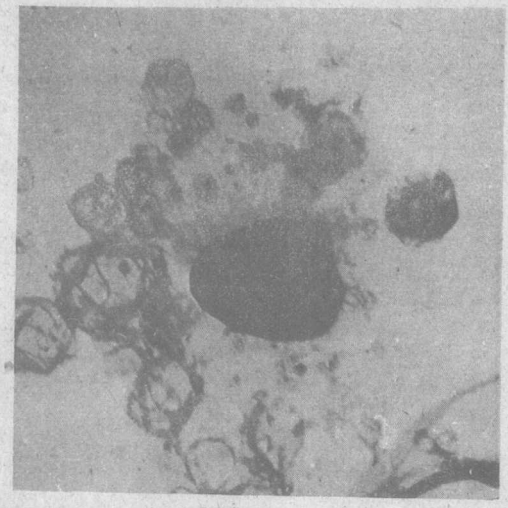

FIG. 3. Trout sperm suspended in distilled water for 30 min. Osmium fixation. Magnification: × 8,500. Electron micrograph by Drs. W Lippert and O. Hug of the Max Planck Institute for Biophysics, Frankfurt/Main.

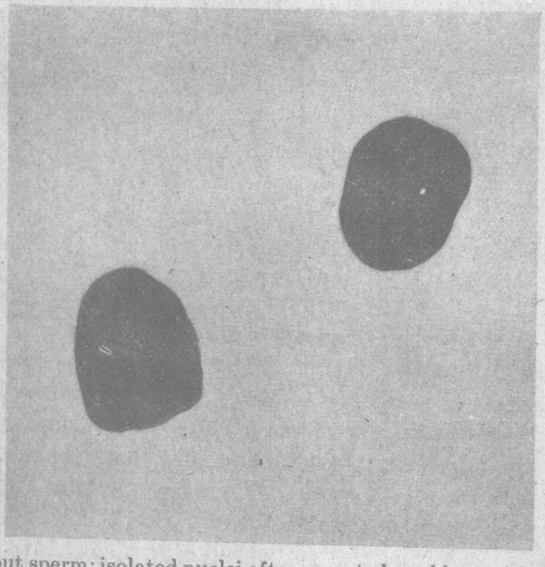

FIG. 4. Trout sperm: isolated nuclei after repeated washings with distilled water. Osmium fixation. Magnification: × 9,000. Electron micrograph by Drs. W. Lippert and O. Hug of the Max Planck Institute for Biophysics, Frankfurt/Main.

b. Method. The best starting material is freshly drawn milt, adequate amounts of which may be obtained from trout in fish hatcheries. Care must be taken to avoid contamination with blood or feces. Two hundred trout yield about 180 ml of milt. If it cannot be used immediately, the milt may be stored overnight in a cold room without loss of mobility of the sperm. The total amount of milt collected is added, with stirring, to about 4 liters

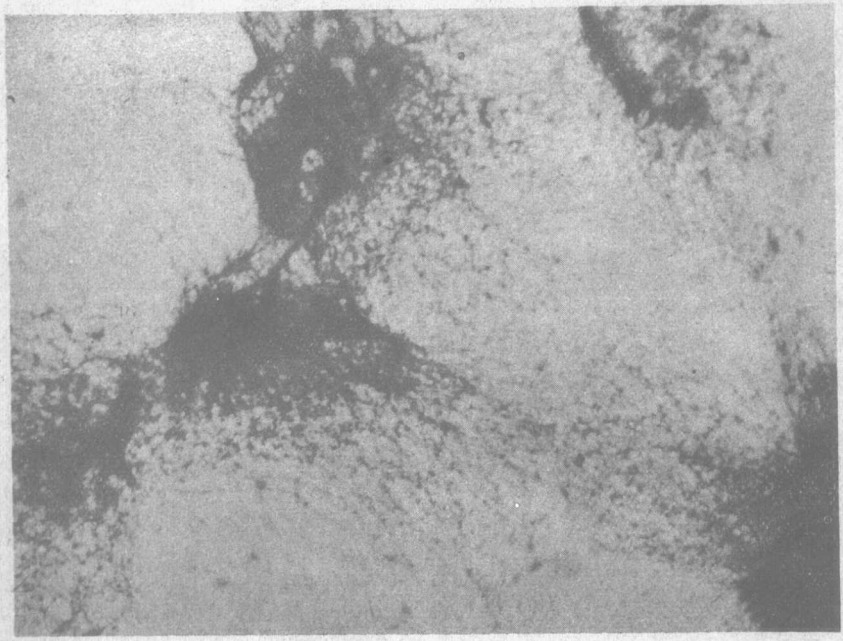

FIG. 5. Trout sperm: nuclei treated on the blend with 10% NaCl and subsequently washed with distilled water. Magnification: × 6,000. Electron micrograph by Drs. W. Lippert and O. Hug of the Max Planck Institute for Biophysics, Frankfurt/Main.

of ice-cold distilled water and cytolysis is allowed to proceed for about 20 min. The supernatant opalescent liquid is somewhat yellowish, probably due to lactoflavine, while the very loosely packed sediment is pure white and consists almost exclusively of the nuclei. It is taken up in 500 ml of water and evenly dispersed, strained through several layers of muslin, and then homogenized in small portions for about 3 min. The combined homogenates are centrifuged at 3000 rpm. The pellet of loose nuclei is repeatedly washed with distilled water and centrifuged until the washings no longer give a reaction for tryptophan. This procedure yields very pure nuclei; however, the losses are fairly large, since the supernatant always