EXPERIMENTAL STUDIES OF PERCEPTUAL PROCESSES

Progress Report, September 1965

SECTION ONE

Covering Section, Budgets

SECTION TWO

I. Complex discriminative behavior in chimpanzees.

II. Fixed ratio reinforcement of large units of behavior.

III. Deferred reinforcement.

SECTION THREE

I. Signal detection psychophysical research.

II. Signal detection in the design of operant experiments.

III. Other research.

SECTION FOUR

Neurobiological program.

Performed under Grant NsG-450 from the

National Aeronautics and Space Administration

INSTITUTE FOR BEHAVIORAL RESEARCH

SILVER SPRING, MARYLAND

EXPERIMENTAL STUDIES

OF PERCEPTUAL PROCESSES

Progress Report, September 1965

SECTION FOUR

Prepared by

ROBERT G. GRENELL, Ph.D.
JOHN THOMAS, Ph.D.
ALAN STUBBS, B.A.
ISRAEL GOLDIAMOND, Ph.D.

NEUROBIOLOGICAL PROGRAM

Performed under Grant NsG-450 from the

National Aeronautics and Space Administration

INSTITUTE FOR BEHAVIORAL RESEARCH

SILVER SPRING, MARYLAND

NEUROBIOLOGICAL PROGRAM

The general neurobiological program is concerned with a specification of particular neurochemical correlates of such behavioral phenomena as learning and memory. A general laboratory for research programs with rats has been established in connection with the Section of Neurobiology of the University of Maryland Psychiatric Institute. The laboratory has recently acquired facilities for housing a large rat colony. The behavioral aspects of this program are carried out at the IBR laboratory and the chemical analyses at the Psychiatric Institute.

I. Behavioral Aspects of Neurobiological Program.

A. Effects of Chemical Substance on Behavior.

A current series of research in the program has involved the use of subjects that have been maintained on a chronic injection program for extended time periods. The rats have been injected chronically with TRIAP, a substance known to alter RNA in neurons and glia. These rats are trained on several different behavioral baselines and their terminal performances are compared to normal subjects on the same baselines. Approximately 20 rats, maintained on the TRIAP injections, were magazine trained and then trained to bar press for food pellets in a standard operant conditioning apparatus. The majority of the subjects were then trained on schedules of reinforcement involving temporal control. The subjects were trained on variable interval or fixed interval schedules, with mean values of from 15 seconds to 2 minutes. The subjects were maintained

on these schedules for several months. The development and maintenance of temporal discrimination on the FI schedules were followed closely. Fine grain analysis of temporal discrimination was obtained through quantitative measurement of FI performance. (Gollub, 1964). Several of the subjects were trained on a visual light intensity discrimination. The rats performed on a multiple fixed interval-extinction baseline. In the presence of a white light, a fixed interval one minute schedule was in effect. In the absence of the light, responses on the bar had no programmed consequence. All of the above subjects have been continually compared to performance of normal rats under approximately the same schedule controls.

Several of these rats have been sent to the Psychiatric Institute for neurochemical analysis. Measurements are also now being carried out on rats injected with TRIAP which have not been subjected to the behavioral experimental situation. The results of behavioral performances, so far, have shown no marked differences between the subjects maintained on TRIAP and normal untreated rats. The chemical measurements, now being carried out, however, may show neurochemical changes or differences that are "behaviorally silent".

B. Effects of Behavior on Chemical Substance.

The general plan of this research is to train naive rats on particular behavioral problems and then to measure changes in RNA in specific neurons and glia, as this may relate to the neurochemical correlates of behavior. The first phase of this research involves discrimination training in a single modality.

For example, rats are trained on a visual discrimination involving light intensity. Tones will also be presented to the subjects, but as irrevelant stimuli. Neurochemical analysis will be made of visual brain areas following training as well as auditory areas and comparisons made between these areas. Other subjects will be trained on auditory discriminations, with different light intensities presented as irrelevant stimuli. Neurochemical analysis will be made of auditory and visual brain areas.

Other subjects are being trained on a discriminated avoidance baseline (Ulrich, et al., 1964) which allows a specific degree of control over the occurrence of behavior. With this baseline, rate of avoidance behavior can be manipulated independently from the frequency of occurrence of aversive stimuli by the appropriate scheduling of conditioned aversive stimuli. Changes in response rate on this general type of avoidance procedure have been previously related to physiological and endocrine changes. Different subjects are exposed to different baseline parameters to produce different response rates. These rats are then sent to the Psychiatric Institute where neurochemical analyses will be made of general limbic system areas as well as other areas.

II. Neurochemical Aspects of Program.

The neurochemical techniques used at this time are several, but major initial effort is being made in the direction of analysis of RNA and its base ratios. Such measurements are now being carried out on rats injected over several generations with TRIAP. The details of the general technique are as follows:

The determination of nucleic acid content and base composition by the biochemical methods is performed on specific cellular structures isolated by microdissection. The nucleic acids are extracted from these structures and analyzed under defined physicochemical conditions -- the essential principles of biochemical work. For the determination of nucleic acid content (Edstrom, 1958; Pigon and Edstrom, 1959; Edstrom and Kawiak, 1961), this approach has necessitated some sacrifice in sensitivity as compared with that attainable with direct photometric measurements on histological sections, but has the distinct advantage in that absolute amounts can be quantitatively determined. The methods are nevertheless suitable for work on the single cell scale (with particularly favorable material even the single gene scale).

Fresh or frozen-dried tissue, advantageous in some cases to minimize postmorten artifacts, may be used by the present methods. Usually, fixed tissue is preferred as it is possible with suitable procedures to preserve the nucleic acids quantitatively and it is advantageous to be able to resort to simple preparative procedures as well as to store the material in paraffin blocks.

For the determination of nucleic acid base composition, microelectrophoresis is employed (Edstrom, 1956, 1960a). The technical basis of the electrophoretic separation of nucleic acid components in small amounts is the reduction in length, width, and thickness of the supporting medium, with the traditional basis for the evaluation of the individual compounds, ultraviolet light absorption, being retained. This means that the increased sensitivity is

obtained by measuring the absorption in smaller areas. However, scaling down the electrophoretic procedure by reducing the dimensions must be accompanied by certain modifications in technique because of the resulting steepening of the concentration gradients of the migrating compounds. Compared to traditional systems the concentration changes per unit length are increased due to the action of two factors: (1) the reduction in linear dimensions causes an inversely proportional increase in the slope of the concentration gradients; and (2) the concentration (weight of the migrating compound per unit volume of the supporting medium) is increased in proportion to the reduction in thickness of the supporting medium. Combined, these two factors give rise to gradients which are of the order of 25,000 times steeper than in traditional zone electrophoresis. This is the main methodological consequence resulting from the reduction in linear dimensions in electrophoresis, and the microelectrophoretic technique consists, in principle, of the practical measures for handling and preserving such gradients. The term microphoresis was introduced for electrophoresis in a scale involving bases and nucleotides in amounts of about 10^{-10} gm (Edstrom, 1956).

The methods whereby ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) content are determined in microscopic tissue units may perhaps be considered as side products of the elaboration of the microphoretic technique. In these cases, the optical density of the extracts in ultraviolet light is determined directly after they have been evaporated to dryness and redissolved to form lens-shaped droplets.

A. Methods

1. Preparation of the Biological Material.

Embedded material can be stored for months without any measureable fall in RNA content. For the isolation of cellular units, sections of a suitable thickness are cut (thick enough to include whole structures, i.e., up to 100-120μ). The sections are mounted on glycerol-egg albumen (1:1)-coated cover glasses (12 x 30 x 0.17 mm) and flattened by heat. The use of water is avoided. The cover glasses can be stored in labeled test tubes or fastened with two strips of tape onto a conventional glass slide, on which identification data can be applied.

Prior to dissection with the micromanipulator, a section is deparaffinized with chloroform for 5 minutes and transferred to successive baths of 100% ethanol and 0.01 N acetic acid, 5 minutes in each. One must blot the hydrated section to remove excess acetic acid. This leaves the section fully hydrated and covered with a thin liquid layer. In the case of units previously isolated free-hand and placed on cover glasses, and which one wants to arrange further with the micromanipulator, it is necessary to use a micropipette to wet the units individually.

2. Micromanipulation

A cover glass with a section or other biological material is placed, with the material facing downwards, over a groove in a thick slide. A space is formed which is surrounded on four sides by the groove and the cover glass and is accessible from two opposite sides. This space is filled with liquid paraffin (Paraffin

liquid for injections, E.Merck A.G., Darmstadt, Germany, No. 7162) which is held in place by capillary force. This arrangement has been adopted from de Fonbrune (1949) and is called an oil chamber. Arranging a cover glass over the groove without any liquid paraffin forms a dry chamber, which is often useful. Finally, one may combine in the same grooved slide a chamber filled with liquid paraffin and a dry chamber (combined chamber). In such a case it is necessary to keep the two cover glasses apart, leaving about a millimeter between the edges to prevent the paraffin from spreading from one to the other.

For isolating tissue units from a hydrated section in an oil chamber, two dissecting needles (see Appendix 2.1) directed by the de Fonbrune micromanipulator (Etablissements Beaudouin, 1 et 3 rue Rataud, Paris 5c) are used. The microscope used in combination with the micromanipulator is a Zeiss Standard microscope equipped with phase contrast optics, phase contrast usually being preferred to other kinds of optical systems during the micromanipulation.

The oil chamber can be conveniently extended with a second clean cover glass marked on the upper side with India ink, on which to place and keep isolated samples. Small hydrated tissue pieces become more transparent after a time in the oil chamber, due to the loss of water. It is therefore essential to have their locations well marked. In other respects the drying is advantageous since it permits the storing of the isolated samples.

For work with two needles attached to the same micromanipulator receiver, the left hand is used for the gross vertical adjustment

screw on the stand of the receiver to change the vertical position of both needles, and the right hand to move the manipulator lever for three-dimensional control of the movable needle. The second needle, connected to the encasement of the receiver and which cannot be manipulated independently, is mainly used for holding.

3. Extraction

Micropipettes can be used in the oil chamber and can be operated by a connection via a rubber tube to a 2-ml air-filled syringe with a two-way stopcock. The second outlet of the stopcock permits the intake or ejection of air (see Appendix 3.1). Two kinds of pipettes are used, one for extractions and hydrolysis and another for obtaining measured volumes. The former kind, with a tapering distal end, is used when only the order of the volumes handled need be known, in which case a rough volume calibration can be done. A column of oil is expelled into a drop of water or glycerol and the diameter of the resulting spherical oil drop is measured, as well as the length of the oil column in the pipette. For standardization and testing of various procedures it is often necessary to transfer accurately determined volumes for which a pipette with a distal nontapering part of several hundred microns and a diameter of 10-12u is used (see Appendix 2.2).

Prior to the enzymic extraction of RNA, the free nucleotides must be removed. Units that are isolated from fresh tissue or collected freehand onto glass cover slips are treated with cold 1 N perchloric acid (0°-4°C), usually for 5 minutes. The perchloric acid is removed with 0.01 N acetic acid in three changes during 5

minutes under agitation. The cover slip is then transferred to a test tube containing 96% ethanol, in which it is stored. Prior to use the material is treated with chloroform, absolute ethanol, and 0.01 N acetic acid as described for tissue sections. It is not necessary to include a special treatment for removing free nucleotides when using fixed tissue since it has been found that these are eliminated during the hydration of the tissue for the micromanipulation (Edstrom, 1953).

Much attention has been devoted to the question of the suitability of Carnoy-fixed tissue for histochemical tests since the control solutions (solutions without enzyme; more properly containing inactivated enzyme) may result in the removal of some RNA (Stowell and Zorzoli, 1947). In the present microchemical work this is unimportant. The way in which RNA is removed when it is collected for analysis is not crucial in itself, but it is imperative that a quantitative removal take place, both for the determination of absolute amounts and for the base analysis. In the latter case, it must be recognized that ribonuclease hydrolyzes the different nucleotides from RNA at different rates. The fact that pancreatic ribonuclease leaves a nondialyzable core rich in purines, has not been found in several tests to prevent complete extraction (Edstrom, 1953; Lagerstedt, 1956, Sandritter et al., 1957; Edstrom et al., 1961). It is desirable to make the extraction as specific as possible, i.e., to reduce the extraction of non-RNA constituents. In a quantitative investigation it was found that extraction as recommended by Brachet (1940), i.e., ribonuclease in distilled water at pH 6.0, is ideal in this respect and much superior to an

electrolyte-containing solution at pH 7.6 which removes some protein from fixed tissue (Edstrom et al., 1961). Unfortunately, the former solution is not always efficient for complete RNA removal when used in small volumes like those employed for microextractions. On this account and because some contamination with the relatively lower ultraviolet-absorbing protein can be tolerated both in determinations of nucleic acid content and for electrophoresis, a buffered solution is usually employed. In some kinds of material where the concentration of RNA is low, ribonuclease in distilled water at pH 6.0 may have particular value (Edstrom et al., 1962). For microphoresis it is necessary to obtain RNA extracts free from buffer electrolytes. This is achieved by using volatile electrolytes in the ribonuclease solution used for extractions (see Appendix 1.3).

Isolated tissue samples placed in an oil chamber are incubated in droplets of enzyme solution about ten times their volume. A large supply drop of enzyme solution is placed in the oil chamber by means of a capillary pipette bent at the tip and operated by mouth. Droplets of the desired size for incubation are removed from the supply drop by the micropipette and placed near each sample. After the micropipette has been emptied, it is used to transfer these individual droplets, each to its sample. The oil chamber is now placed in a Petri dish, the bottom of which is covered with a filter paper moistened with a few drops of the volatile buffer, and is then kept in an incubator at 37°C for 30 minutes. After this time a new supply drop of enzyme solution is introduced into the chamber. New volumes for the second extraction are taken

with the micropipette from this drop and placed near each incubated object. A dry cover glass is also added, giving a combined chamber. Each original extract is then individually transferred to the dry cover glass where it is evaporated to dryness. After the delivery of an extract the pipette is used to transfer the prepared second incubation volume to the extracted cell. In this way the pipette is washed and the washings included in the following extract. A second digestion at 37°C for 30 minutes is then carried out, after which extracts are evaporated to dryness and new volumes of enzyme for the third extraction applied as after the first incubation period. Three extractions are usually performed. Extracts from the same sample are evaporated close together. During the incubations in a humid dish the dry cover glass is removed from the chamber. The times are doubled for formalin fixed tissue.

For determination of nucleic acid content the pooled and dried extracts should be kept within as small an area as possible. Extracted tissue units may be stained afterward for control of the digestion, identification, etc., by removing the paraffin with chloroform and drying the glass. If the glass is placed in a test tube with a hole in the bottom, it is conveniently handled in a staining procedure.

4. Hydrolysis

Prior to an electrophoretic separation, the extracted nucleic acid is hydrolyzed with hot 4 N HCl to liberate the purine bases and pyrimidine nucleotides from ribopolynucleotides.

The problem of maintaining small volumes of aqueous solutions at high temperature is solved by performing the hydrolysis in a micropipette of the type that is used for extractions. In such a pipette, 4 N HCl is first introduced as a column several millimeters long followed by a volume of liquid paraffin large enough to give a safe separation between the long column of hydrochloric acid and the hydrolyzate which is to follow next. The extract is dissolved in a volume of 4 N HCl, in which the concentration of RNA in the hydrolyzate does not exceed about 1%. After the hydrolyzate a series of small alternating volumes of liquid paraffin and 4 N HCl is introduced into the tip to prevent evaporation of the hydrolyzate and enable transportation of the pipette for hydrolysis. The hydrolysis is done at 100°C in a bath of liquid paraffin saturated with 4 N HCl. A cylindrical vial (weighing tube) of 80 mm height and 35 mm diameter is filled two-thirds with liquid paraffin and the bottom covered with a layer of 4 N HCl, 0.5-1 cm high. The vial is shaken and placed overnight in an oven at 100°C. It is taken directly from the oven to a boiling water bath. The pipette is mounted in a slit of a rubber stopper and inserted into the vial so that the tip reaches the lower half of the liquid paraffin. After 30 minutes the hydrolyzate is extruded in an oil chamber (the plugging volumes of HCl may be included in the hydrolyzate) and afterward evaporated to dryness using a combined chamber. When used for electrophoretic analysis the dry hydrolyzate is dissolved in a minimal volume of 4 N HCl in an oil chamber. As a guide to finding

a suitable volume, it is recommended to take about twice the volume necessary to dissolve the substance. Dried hydrolyzates must not be stored under liquid paraffin, since this may cause a loss of purines.

In preference to 1 N HCl, traditionally used for hydrolysis (Smith and Markham, 1950), 4 N HCl was chosen. In contrast to the former, it dissolves RNA extracts quickly at room temperature. For hydrolysis of RNA 1 N HCl is generally used for 60 minutes at 100°C. The use of 4 N HCl for only 30 minutes compensates for the increased acidity but may be a somewhat stronger treatment. However, in quantitative tests it has not been possible to measure any difference in results of the two hydrolytic procedures (Edstrom, 1960a).

5. Electrophoresis

Electrophoresis on a microscopic cellulose fiber is employed to separate the RNA constituents in amounts of 10^{-10} gm (i.e., 500-1000 μμg RNA) from each other. The medium in which the electrophoretic migration occurs consists of two phases, one of which is the supporting framework of cellulose molecules, and the other a solution, the electrophoretic buffer, in which the applied compounds dissolve. This system gives the compounds a specified ionization and allows the migration of ions in an electric field. In the microphoretic work the electrophoretic buffer is characterized by certain properties prerequisite for obtaining sharp separations on a microscopic scale. These properties are a high viscosity (4000-5000 cp); a relatively high conductivity, approximately $0.7 \cdot 10^{-3}$ mho/cm, and for the special case where purines are involved, a high

acidity (around pH 0) to increase the solubility of these compounds. The basis for detection and determination of the microphoretically separated nucleic acid constituents is their absorption of ultraviolet light at 257 mμ. It is essential that the electrophoretic buffer has no more than a moderate absorption at this wavelength. An electrophoretic buffer meeting the specifications enumerated above is prepared from sulfuric acid, glycerol, and glucose as described in Appendix 1.8.

The supporting cellulose framework is obtained from artificial silk produced according to the cuprammonium method, designation: "Cupresa HW Naturglanz, ungedreht im Strang," 0.8 den. per filament (Farbenfabriken Bayer A.G., Dormagen, Germany). The individual fibers of the silk thread are used after they have been induced to swell by treatment with alkali (see Appendix 1.7). In an untreated state they have a diameter of 9μ and in the final state, during electrophoresis, 25-30μ. After treatment, the fibers are kept in the electrophoretic buffer.

To isolate individual fibers from the bundle of entangled fibers obtained after the alkali treatment, one first obtains a free fiber end by pulling the bundles apart with a needle. Such an end may be taken by rotating it onto the tip of the needle. It is slowly pulled free of the other fibers and placed onto a clean glass slide, with one free end protruding over the edge of the glass. Excess electrophoretic buffer is removed by transferring the isolated fiber successively to new parts of the slide, whereby a wet track of decreasing

thickness is left each time. After six or seven such transfers, the fiber is usually ready for use as evidenced by thin and uniform tracks resulting from the later transfers.

For microphoresis the fiber is placed, slightly stretched to keep it straight, on a quartz glass, 25 x 30 x 0.5 mm (obtained from W.C. Heraeus GmbH., Hanau, Germany), parallel to the longest side. A fiber length of 12-20 mm and a diameter of 25-30μ is suitable. Buffer bridges are applied from the ends of the fiber toward the long edge of the quartz glass, the bridges ending about 5 mm from the corners with a hook over the edge. The buffer bridge is used in the form of a paste made by mixing the electrophoretic buffer with finely powdered SiO_2 to a consistency such that it can be smeared out but will not flow out along the fiber. The fiber is then ready to be used for the application of hydrolyzates. The quartz glass, with the fiber underneath, is placed behind a cover glass with hydrolyzates in an oil chamber to give a combined chamber. The microscope is equipped with a small heater used during the application of hydrolyzates (see Appendix 3.2). While initially heating the fiber for a few minutes, suitable application points for hydrolyzates are marked by applying droplets of liquid paraffin with the micropipette to the quartz glass close to the desired application points. Although the fiber is not even along its whole length, several uniform segments (one separation requiring about 500μ) are usually available on a given fiber. The markings are made at places where the fiber shows an even diameter for at least 500μ in the direction of the cathode. Hydrolyzates are applied by touching

the lower side of the fiber lightly with the tip of the micropipette, applying to the syringe a slight positive pressure which is increased as the column of hydrolyzate is shortened. The pipette is emptied as completely as possible of the hydrolyzate. Often some liquid paraffin flows out and spreads along the fiber, but paraffin in such small quantities is not apt to cause any inconvenience. The hydrolyzate should stay as a slightly bulging concentrated spot at the point of application. It takes 5-10 seconds to apply an extract to the fiber, which is used for several hydrolyzates as a rule. It is usually possible to find six or seven suitable locations for separations on a fiber of 15 mm length.

After the application of hydrolyzates, the fiber is in a relatively dry state and the viscosity of the electrophoretic buffer is far above the required one. The correct viscosity in the fiber is obtained by exposing it to an atmosphere of 42% relative humidity for 5 minutes in a constant humidity chamber specially constructed for this purpose (see Appendix 3.4). The quartz glass is introduced into this chamber without disturbing the relative humidity and brought into contact with the electrodes. After 5 minutes in the chamber, equilibrium has been reached and the electrophoretic buffer has absorbed the amount of water required to give the right viscosity for successful separations. The fiber is covered with liquid paraffin while in the chamber and 2 kv applied for a time that is proportional to the fiber length (about 0.7 x fiber length in mm expressed in minutes, i.e., 8-14 minutes for a fiber 12-20 mm long).

A current of the order of 10-μa is obtained at 2 kv measured with a "Scalamp" galvanometer, model 7904/S (W.G. Pye & Co., Ltd., New Market Road, Cambridge, England). An extra high power supply is used delivering 2 or 4 kv (see Appendix 3.3).

6. Photography and Photometry

After complete electrophoretic separations have been obtained, the quartz glass is taken to the ultraviolet microscope (see Appendix 3.5 and 3.6) for inspection and photography at 257 mμ.

Photography is carried out with a low power lens (16-mm quartz monochromate, corrected for 275 mμ, N.A. 0.20) and a 10X quartz ocular. A fluorescent eyepiece is used for focusing. A bellows length of 25 cm providing a total magnification of 93X is used. It is not necessary to include regularly a reference system since the absorbing bands are evaluated by comparison with each other.

Certain precautions must be taken during photography. Excessive illumination of the fiber exerts a selective destructive action on uridylic acid and a consequent reduction in its optical density (OD) at 257 mμ. Consequently, a standard time of 10 seconds illumination has been introduced for all focusing before photography, and the exposure time for the plates has been standardized to 30 seconds. The time for film exposure will of course vary depending upon the type of illumination; a time has to be selected by trial which will allow the absorbtion by the four separated bands to fall within the linear part of the photographic blackening curve. The characteristics of

the latter may be determined with the aid of a reference system (see Appendix 3.7). Kodak O 250 plates are used, developed for 2 minutes in a Kodak D72 developer, afterward rinsed with water, and fixed with a Kodak F52 solution.

The exposed plates are scanned in the recording microdensitometer described by Walker (1955) (Joyce, Loebl & Co. Ltd., A8, Princesway, Team Valley, Gateshead on Tyne 11, England). The picture of the fiber is scanned with a 16-mm objective along its length at an arm ratio of 1:2 and with a gray wedge extending from 0.5 to 2.5 OD units. A slit covering about half the diameter of the fiber is used for the scanning beam of light. The background along the separation is recorded as close to the fiber as possible.

7. Calculations

Before integrating the areas under the peaks of the curve, a base line is drawn parallel to the background tracing through parts of the curve representing nonabsorbing segments of the fiber. The areas under the peaks are evaluated from the weight of copies prepared on transparent paper. Since in microphoresis only the quotients between the integrated areas are of interest, it is sufficient to determine their relative size. (For calculations of quotients see Appendix 4.1). The reference OD values have been taken from the absorbtion curves published by Beaven et al. (1955). For the purines the values are based for solutions in 6 N HCl. In quantitative tests it has been found that the use of these constants gives a recovery close to 100% (Edstrom, 1960a). Hot acid hydrolysis gives approximately 5% dephosphorylation of the pyrimidine nucleotides (Markham and Smith, 1951).

For uridylic acid this is unimportant since uridine stays at the same place as the nucleotide and has the same absorption properties. Cytidine migrates faster than cytidylic acid but occupies a place that lies closer to cytidylic acid than to guanine. Unless very extended separations are used, the similarly absorbing cytidine will, therefore, be included with cytidylic acid.

8. Determination of Nucleic Acid Content

A relatively simple method is available for the determination in microscopic tissue units of the RNA content in amounts down to 25 μμg. The ribonuclease extracts are collected on a quartz glass in a combined chamber. The quartz glass, of the same kind as used for microphoresis, is pretreated to remove all water from its surface. (Storing of the cleaned and dried glass in a dry organic liquid like chloroform or decane for a couple of days.) It is necessary to remove the quartz glass from the oil chamber during the digestions of RNA in a humid dish as described in Section II, A.3. The quartz glass with the collected RNA extracts is used to make an oil chamber. The extracts, when dissolved in a glycerol-containing buffer (see Appendix 1.5), form round lens-shaped drops of a regular outline. To decrease the contact area between the pipette tip and the drops, a pipette with an angle of 45°-50° toward the horizontal plane and a tip diameter of only 6-7μ is used for the applications of the glycerol-containing buffer. The nucleic acid spots on the quartz glass are photographed in ultraviolet light at 257 mμ with a reference system giving steps with differences in optical density of 0.1505 (at a magnification of 130X). A fluorescent eyepiece is used for focusing.

The pictures of the spots are investigated by photometry at a further magnification of 2, 5, or 10X, depending on the size of the spots. Two tracks are run through the center at 90° to each other and superimposed. A mean curve is drawn on transparent paper, and it is then possible with the aid of a device for integration of the absorption (see Appendix 4.3) to determine the amount of RNA in the spots (See Appendix 4.2). This method also works for DNA extracted with deoxyribonuclease (Edstrom and Kawiak, 1961) (see Appendix 1.4). In this case four extractions, 1 hour each, are performed at 37°C.

B. Accuracy

The microphoretic method has been scrutinized in several tests for recovery and accuracy. The method should be checked with RNA of known composition, such as yeast RNA, from time to time. As an aid to other in locating artifacts, some potential sources of error will be pointed out. Incomplete extraction tends to increase the ratio of pyrimidines to purines. Incomplete hydrolysis (which may result from the use of unsaturated paraffin in the bath or too small volumes of hydrochloric acid for the hydrolyzate) increases the absorption at the position of cytidylic acid, since unhydrolyzed purine compounds will be localized here and measured as cytidylic acid. Incomplete delivery of an extract from the micropipette may give rise to a transfer of varying efficiency for different hydrolysis products. Extracts contaminated with large amounts of proteins give increased absorption at the application point, i.e., at the position of uridylic acid. This error can be lowered by storing the isolated material in 96% ethanol for several days. Low uridylic acid values are obtained

by excessive illumination in ultraviolet light. A small spectral bandwidth of the light during photography is important for quantitative work, but this source of error is easy to avoid in ultraviolet microscopes utilizing illumination with a discontinuous spectrum, such as the Kohler ultraviolet microscope. On account of the differences in the OD of the separated bands, care must be taken that the linear part of the photographic blackening curve is utilized and that due regard is paid to stray light. Finally, unsharp and extended zones may arise from several sources, the most important of which are (1) overloading of the fiber with hydrolysis products, (2) insufficiently swollen fibers, (3) defective heating during the application of extracts, and (4) lag of time between the completion of a separation and photography.

There is a random error between individual analyses within a hydrolyzate as well as between different hydrolyzates from the same material, of which the latter variation is of greater interest. For biological material an average coefficient of variation (V) between hydrolyzates of 5% was found by Egyhazi and Hyden (1961) and 7% was determined by Edstrom (1956) on yeast RNA.

An analysis of the different steps in the procedure for determination of RNA content shows that the errors involved are quite low (Edstrom, 1953, 1958). The photographic-photometric part of the procedure has been tested with measured amounts of RNA. The values obtained in this way show a quantitative recovery and a V-value of about 5%. An idea of the total random error in the method may be obtained by using homogeneous biological material. Determination of

the RNA content in single nerve cells from the cochlear ganglion cells of the guinea pig gives values with an average V-value of 12.7%. By analyzing groups of ten cells the biological variation is reduced by a factor of $1/\sqrt{10}$. From the average V-value for group data, the random error of the method can be calculated. The results show that 4.1% of the variation is due to the method, 12.0% being the V for the biological variation between single cells.

The method for the determination of total amounts of RNA is easier to check because more favorable material is available, such as nondividing cell nuclei. It seems likely that spermatocytes and spermatids show an insignificant biological variation. With these cells as well as groups of calf thymocytes and rat spermatids, V-values of 6-10% were obtained (Edstrom and Kawiak, 1961). Mean values for calf thymocytes of 7.7-8.1 μμg were measured. Since biochemical analyses (for reference, see Davidson, 1950) have given values of 6.5-7.5 μμg per nucleus, the possibility exists that this method contains a positive systematic error of 10-15%, although an analysis of the method failed to show any cause for such an error.

C. Applications

The nucleic acid methods have found a special application in two fields: the study of the RNA of different cellular organelles and chemical studies on the central nervous system.

A consistent finding has been the similarity in base composition between nucleolar and cytoplasmic RNA (Edstrom, 1960b; Edstrom et al., 1961; Edstrom and Beermann, 1962), a finding that agrees with

radioautographic and other evidence for a nucleolar origin of at least a part of the cytoplasmic RNA (see Perry et al., 1961). In the nucleus, in addition to the nucleoli, nuclear sap and chromosomes may be separately analyzed in suitable material (Edstrom and Gall, 1963). In the giant chromosomes of the chironomids it is possible to analyze pooled Balbiani rings, which are products of the activity of single genes (Beermann, 1961), to obtain information on the base composition of their RNA (Edstrom and Beermann, 1962).

The nervous system is a tissue in which the need for microchemical procedures is particularly evident because of its complex morphology and asociated complex function. Evidence that the central nervous system possesses biochemical differentiation of a similar order of complexity has been accumulating for several years. As part of broader metabolic studies of the nervous system, Hyden and co-workers have been using the microchemical methods for the determination and analysis of RNA. In functional and pharmacological studies it has been found that neurous and glia demonstrate quite specific changes in RNA content and composition after various kinds of treatments (Egyhazi and Hyden, 1961; Hyden and Egyhazi, 1962). The fact that the neuronal RNA is comparatively resistant to postmortem changes (Jarlstedt, 1962) and that fixed and embedded material can be used is of value for investigation of clinical material. Gomirato and Hyden (1963) found that RNA in glia of basal ganglia is grossly abnormal in patients with Parkinson's disease.

D. Concluding Remarks

Although microphoresis has to the present been used mainly for the base analysis of RNA, it is evident that several other applications remain. Thus it can be used with advantage for the determination of the adenine:guanine quotient in DNA (Edstrom and Beerman, 1962). A combination of microphoresis and radioautography may prove particularly useful. An enzymological application by Sierakowska and Edstrom (unpublished) on the ribonuclease and phosphatase activities in subcellular parts of starfish oocytes has commenced. Subcellular parts isolated in a nonpolar medium are placed in the oil chamber into small incubation droplets containing suitable substrates (cyclic cytidylic acid for ribonuclease and noncyclic nucleotide for phosphatase). After incubation the contents of the droplets are analyzed by microphoresis, and enzyme activity is revealed by the presence of decomposition products.

One may want to know whether still greater sensitivity of the microphoretic technique is to be expected. Theoretically the lower limit is set by the solubility of the migrating compounds in the impregnated silk fiber. With the use of ultraviolet absorption for detection and measurement and on the assumption of a minimal efficient OD of 0.3 for the separated bands, a maximal solubility of 10 mM and an optical molecular density per centimeter (e) of 10,000, it can be calculated that a cuvette depth of 33u is necessary. The most sensitive procedure and the only one currently feasible is to use the supporting medium as a cuvette during the absorption measurements. Using a medium of cylindrical shape (fiber) and assuming an efficient zone length of

twice the diameter, it can be calculated that an amount of about 1 uumole can be separated and determined. This figure gives only the order of the sensitivity, in practice it has been possible to obtain reasonably good separations involving nucleic acid components in amounts corresponding to about half of this value. It is evidently not possible to obtain a fundamental improvement in sensitivity by applying the present principles for the separations. For this purpose it is necessary to modify the procedure in an essential aspect, e.g., by sharpening the bands (to give discs), or using more sensitive methods of quantitation (e.g., measurement of induced fluorescence or radioactivity).

With regard to the determination of nucleic acid content, it seems reasonably possible to obtain a higher sensitivity by adapting the shrinking droplet technique of Ornstein and Lehrer (1960).

E. Appendies

Appendix 1: Solutions and Chemical Treatment

1.1. Carnoy Fixation - The following procedure is recommended for tissue pieces 2-3 mm thick: Immersion in freshly prepared fixative according to Carnoy (absolute ethanol-chloroform-glacial acetic acid, 6:3:1, by volume) for 90 minutes at room temperature, followed by absolute ethanol and benzene, 90 minutes each and infiltration with paraffin for 4-18 hours.

1.2. Formalin Fixation - Tissue pieces, 2-3 mm thick, are fixed for 3 hours in Lillie's buffered formalin (100 ml commercial formalin, i.e., 37-40% formaldehyde; 900 ml distilled water; 4.53 gm $NaH_2PO_4 \cdot 2H_2O$ and 7.23 gin $Na_2HPO_4 \cdot 2H_2O$) at room temperature, followed by running tap water overnight, dehydration in 96% and absolute ethanol, 90 minutes in each, benzene and paraffin as above.

1.3. Ribonuclease Solution with Volatile Electrolytes - Dissolve 0.4 mg of pancreatic ribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) in 1 ml 0.2 M ammoniumbicarbonate-acetate buffer, pH 7.6. The latter solution is prepared by dissolving 1.58 gm ammoniumbicarbonate in 100 ml distilled water and adjusting with 0.2 M acetic acid to pH 7.6. It is stored in the refrigerator after addition of a few drops of chloroform. The pH is checked before use and adjusted with acetic acid if necessary. Freshly dissolved enzyme is used.

1.4. Deoxyribonuclease Solution - Pancreatic deoxyribonuclease (Worthington) is used in a concentration of 0.4 mg per milliliter 0.02 M phosphate buffer pH 7.0, containing 0.003 M $MgCl_2$, 0.0025 M

hydroxylamine as hydrochloride (not 0.005 M as stated in the original paper, Edström and Kawiak, 1961), and 0.1% gelatin. The buffer solution is kept in the refrigerator and should be renewed monthly. Only freshly dissolved enzyme is used.

1.5. Glycerol-Containing Buffer for RNA - A solution is made of 2 parts by volume of 0.2 M Na, K phosphate buffer, pH 7.6, and 5 parts of water-free glycerol, specific gravity 1.26. The OD at 257 mμ (or the wavelength used for photography in the ultraviolet microscope) is measured in a spectrophotometer in 1-cm cuvettes and compared with the OD of the liquid paraffin used. The OD of the liquids, if not agreeing within 0.3 OD units, should be made equal, e.g., by dissolving adenine in the usually less absorbing buffer.

1.6. Glycerol-Water Mixture for DNA - Since the DNA extracts contain buffer salts, it is sufficient to use a glycerol-water mixture (6:1, by volume) for these. The OD at the wave-length used for ultraviolet photography should be checked and adjusted as for the glycerol-containing buffer for RNA.

1.7. Alkali Treatment of Cellulose Fibers - Cupresa silk thread is cut in about 30 pieces, 2 cm long, which are placed in a beaker with distilled water. They are transferred to 1.5 N NaOH when wet and 2 minutes later to 2.25 N NaOH. The treatment with the latter solution is carried out at 10°C for 5 minutes under gentle stirring with a glass rod. Since the fibers become too soft during the immersion in the 2.25 N NaOH to be lifted without tearing, it is necessary to empty the beaker into a larger volume of distilled water, from which it is possible to lift the fibers with a glass rod to three

subsequent changes of distilled water, 2 minutes each. The fibers, after excess moisture has been removed with filter paper, are finally placed in the electrophoretic buffer and shaken for an hour on a mechanical shaker and then placed in a refrigerator. A preparation can be used from the day of its preparation for a couple of months (it was earlier incorrectly believed that the fibers could be used only during the first 3 days, Edström, 1960a).

1.8. Electrophoretic Buffer - The electrophoretic buffer has been modified since it was first described (Edström, 1956). The sulfuric acid concentration has been increased and the heating time shortened. It is prepared as follows: 20 ml 8 N sulfuric acid, 8 ml distilled water, 33 gm glycerol (specific gravity 1.26) and 72 gm water-free D-glucose are mixed in a flask and heated in a water bath at 100°C under stirring until the sugar dissolves. After 20 minutes the flask is removed, cooled rapidly, and stored at — 20°C, where it will keep for months.

Appendix 2: Microinstruments

2.1. Glass Needles - Glass needles are prepared from 3-mm soda glass which is pulled to give a 5 cm length of 1mm diameter adjoining 3 cm of the thicker piece. The end of the thin part, softened in the de Fonbrune microforge (Etablissements Beaudouin, 1 et 3, rue Rataud, Paris 5e) by contact with the heated platinum wire, is pulled at 50°—60° from the axis to a cone with a blunt tip. The working field is then cooled by applying a stream of air and the electrical heating of the wire increased correspondingly. The needle is brought in contact with the wire a second time but now barely touching the

tip. By removing the needle from the wire a sharp tip can be obtained.

2.2. Micropipettes - The starting material is 8- to 9-mm pyrex glass tubing, maximal wall thickness 1 mm. Care must be exercised during the different stages of pulling that the relative wall thickness is not increased by heating. Capillaries about 1 mm in diameter are first pulled in a hydrogen burner and cut in approximately 10 cm lengths. At 2 cm from one end the capillary is heated with an alcohol frame and pulled to give a short local thinning of about 0.5 mm diameter. A hook is made by heating the capillary near its end, distal to the thinning. The capillary is then introduced into the long arm of a glass holder made of 3-mm tubing with right angle arms of 2 and 4 cm length. The short arm is to be connected to the syringe used for maneuvering the pipette. The holder with the capillary sealed in by picein is inserted in the microforge holder and placed in a vertical position with the hook and the short glass holder arm extending toward the right. A 2.5-3 gm weight is hung on the hook and the glass instrument inclined 25° from the vertical position. The capillary is then heated with the platinum wire (which should not be allowed to touch the glass) about 1 mm above the point where the capillary shows the maximal thinning. After the capillary has bent, the angle is increased a further 10° (to a total of 35°) and heated at its thinnest point, where it is kept at about half the distance of a capillary diameter. Heat is applied in such a manner that the capillary will become extended slowly to a pipette tube of microscopic dimensions and break after 10-20 seconds of heating. A useful shape can be found

by experience. If the micropipette is too long (too much heat) it has inconveniently high resistance during its operation due to capillary forces; if it is too short, it is readily broken. An orifice of 8-10 μ in diameter is suitable for most types of work. A smaller opening, for the application of glycerol-buffer droplets to RNA and DNA extracts, is obtained by using lighter weights.

Satisfactory pipettes for delivering measured volumes (after calibration) are obtained by stopping the heating of the pipette before it breaks, moving it upwards 2-3 mm, and approaching it again with the heated wire until it breaks, this time using the air stream to obtain localized heating. This kind of pipette is given a total inclination of about 10^{o}. To calibrate it, a column of liquid paraffin is trapped between the tip and a column of water behind the paraffin, a very small volume of water also being used to seal the tip. The paraffin column length is measured with an eyepiece micrometer, after which the column is expelled into a droplet of glycerol stained with methylene blue. From the diameter of the sphere formed by the paraffin the volume corresponding to the measured length of the pipette can be calculated.

With the use of an air-filled system an explosive delivery may occur due to the resistance towards expulsion of a volume of water solution suddenly being overcome as the positive pressure increases in the syringe. The use of thin-walled pyrex tubing decreases this tendency; contaminations on the inner pipette surface enhance it. Clean tin-walled pipettes of the shapes and sizes described above seldom

cause difficulties of this kind. For the preservation of good functional pipettes they should be made slightly hydrophobic with liquid paraffin, which is kept in their distal parts for a day before they are used for water solutions. This paraffin is of further use as a column behind the pipetted volumes of aqueous solutions, preventing evaporation and liquid losses toward the rear. It is also essential for proper functioning of the pipette that the meniscus between the paraffin and the water solution is not moved so fast that droplets of any phase are left behind. The pipette is rinsed with 4 N HCl from a droplet in the oil chamber after being used, all aqueous solutions expelled, and the pipette stored with liquid paraffin in its distal end.

Appendix 3: Apparatus and Equipment

3.1. Syringe for Pipetting - The micropipette is connected by means of a rubber tube to a 2-ml "Inaltera" syringe with a three-way stopcock (Georg A. Henke, Tuttlingen, Germany). The stopcock plug is fastened onto a cone-shaped brass stand fixed to a wood platform on which both the microscope and the micromanipulator are placed. The stopcock body with the syringe can be moved around this axis. Use is made only of two outlets, one outlet is for the rubber tube, the other one is left free for the intake or expulsion of air.

3.2. Heater for Drying Fibers and Hyprolyzates - A platinum wire, 6 cm long, 0.6 mm in diameter, and spirally wound,is housed in a metal cylinder, 2 x 6 cm, with an open slit a fourth of its circumference wide except for shorter pieces at the ends, which are left intact. The wire, connected to electrical cables leading to a variable transformer by means of metal adaptors located in the ends of

the cylinder, is insulated from the latter by ring-formed porcelain insulators. The insulators are glued to the cylinders and the adaptors by means of litharge cement. The cylinder, with the opening facing toward the object field, is attached to the microscope stand near the objective nosepiece. The platinum wire is fed from a variable transformer unit consisting of a transformer 220 v/3 v, maximum effect 45 w. It has a fixed resistance of 300 ohm and a variable one of 1000 ohm on the primary side.

3.4. Constant Humidity Chamber -- A chamber (Edström and Pilhage, 1960) providing constant humidity conditions for the microphoretic separations was constructed for this purpose. Chambers, as well as other microphoresis equipment, may be obtained from Rudolph Grave AB, Box 43, Mölndal 1, Sweden.

3.5. Optical Equipment for Ultraviolet Microscopy - Apart from the usual demands on a microscope arrangement for photography in monochromatic light, the microphoretic procedure and the subsequent quantitative determination of the ratios of the separated compounds impose certain restrictions on the choice of equipment. A practical advantage is to be able to include the whole separation in one plate which requires low power lenses. The area that is reproduced by a 16-mm objective and 10 x ocular is large enough to include a whole separation and enough background. Such a quartz monochromate, corrected for 275 mμ, as used in my work, is made by VEB Carl Zeiss, Jena, Germany. Monochromates corrected for 254 or 275 mμ may also be obtained from Cooke, Troughton & Simms, York, England. The latter firm also provides

a quartz substage condensor with removable front lens for work with the 16-mm quartz monochromate. A suitable eyepiece is a 10 x quartz ocular; a fluorescent eyepiece must be used for direct viewing of the field in ultraviolet light. Both kinds are supplied by the British firm. Before the equipment is set up, it must also be ascertained that the light intensity is such that the exposure times will be moderate. As it is desirable to run simultaneously several separations and as there is free diffusion in the fiber, each exposure has to be so short that all photographic work can be finished within 2—30 minutes after the completion of the separations. (This factor may be of importance if direct photometry in the ultraviolet is tried.)

I have been using as a light source the spark generated between rotating cadmium electrodes used by Köhler (1904). The spectral lines are dispersed by means of two 60° water-filled quartz prisms. The line at 257 mμ, purified by means of a filter according to McFarland et al. (1958) (see Appendix 3.6, is used for the quantitative work. This light source involves motors to rotate the electrodes; a transformer 200 v/5 kv, maximal effect 4 kw; and a converter from 50 to 500 cycle per second. As the cadmium spark generates poisonous fumes it has to be well housed and evacuated. The equipment seems unnecessarily complicated today and complete ultraviolet microscopes with low power optics are now supplied by Cooke, Troughton & Simms, as well as by Carl Zeiss, Oberkochen, Germany. If it is possible to choose wavelengths, 265 mμ is recommended since it is located relatively near the absorption maxima of all four ultraviolet-absorbing compounds. It should be pointed out that particularly in the case of microphoretic

separations, it is essential that the spectral bandwidth of the light used by low since the error in the quantitative evaluation grows at different rates for the different compounds with increasing bandwidth. A lamp generating high intensity ultraviolet light is valuable in combination with a monochromator since it permits a low bandwidth to be used. The illumination should be kept as low as possible also to reduce photodecomposition of uridylic acid. Scott (1955) and Walker (1956, 1958) give detailed information on microscopy and measurements in ultraviolet light.

3.6. Filter for Isolating Ultraviolet Light - A suitable filter for combination with the cadmium light source or a mercury low pressure lamp to purify the lines at 257 and 254 mμ, respectively, has been described by McFarland et al. (1958). Polyvinylalcohol, 98% hydrolyzed (Maltheson, Coleman and Bell, Norwood (Cincinnati), Ohio, USA), is mixed with water at a concentration of 10% (w/v) and heated in a water bath until the polymer dissolves. The liquid is poured onto a glass plate as a film 0.25 mm thick after drying. According to the recommendations of McFarland et al. the film is stained after it has been peeled from the plate, but I have found it easier to stain the film while on the plate. The stain solution consists of 1 gm iodine and 2 gm potassium iodide in 100 ml distilled water. A treatment time of 5 seconds is recommended but this, as well as the concentration of the dissolved compounds, may be varied to give filters of different densities. The relative amounts of iodine and potassium iodide must be kept constant.

3.7. Reference System - A reference system for calibrating blackening may be obtained by using a rotating disc with zones having apertures differing by a constant logarithmic ratio. Since most low power quartz monochromates give rise to a slight amoung of stray light, it is desirable to compensate for this error by means of a rotating disc in the following way: the OD of heavy concentrates of nucleic acid in spots of the size used for microchemical determination is evaluated by comparing their density with a part of the photographic plate exposed for a fraction of the total exposure time. (In the absence of stray light the OD of such spots is infinite.) As an example one may find that such spots have the same blackening as emulsion exposed for one fortieth of the total exposure time. This means that the stray light is 2.6% of the refracted light. The resulting error, small at low OD values, increases with increasing densities. Objects with an OD of 0.5 will be measured with a 5% negative error in this case if no compensation is made. The rotating disc used for calibrating blackening can be constructed to compensate for such stray light. A suitable OD difference between each adjoining zone is 0.1505, i.e., log 2. In an uncompensated system the ratio between each outer aperture to that of its inner neighbor will, consequently, amount to 2. Correction for stray light gives ratios equal to

$$(100 + s): (100/\sqrt{2} + s),$$

where s is the amount of stray light is per cent of the incident light, the measured apparent OD in the uncorrected system of the opaque spot being log $(100 + s): s$.

It is known that a rotating sector may give rise to errors such as an intermittency effect and interference with the intensity variations of the light source (Thorell, 1947). There is no need for a special check on this point since the whole photographic-photometric procedure can be tested directly with measured amounts of nucleic acid.

Appendix 4: Calculations

4.1. Calculations of Base Ratios - The relative areas circumscribed by the peaks of the photometer curve and the base line are determined as previously described for the separated compounds. The values are divided by the corresponding ϵ-value at the wave length in question. At 257 mμ the ϵ-values x 10^{-3} are 11.2, 9.6, 5.15, and 9.5 for adenine, guanine, cytidylic acid, and uridylic acid, respectively (values from the curves published by Beaven et al., 1955). The resulting quotients are added and the percentage of the sum that each quotient constitutes is calculated.

4.2. Calculation of Total Amounts of Nucleic Acid - The formula for the calculation of the amount of nucleic acid in an extract is:

$$x = \frac{\Sigma h \cdot f^2 \cdot s \cdot 0.1505}{a \cdot d}$$

where x is the amoung of nucleic acid in μμg; Σh is the sum of the mean zone heights in millimeters as determined with the aid of the absorption integrating device (half of the sum of values from both sides), see Appendix 4.3; f is the number of microns corresponding to 1 cm of the horizontal dimension of the photometer curve, i.e., 10,000 X the inverted value for the magnification in the photometer curve; s is the value in square centimeters of the central circular area, i.e., the area common to all zones in an absorption integrating

device; a is the height in millimeters of each step of the reference system; 0.1505 is the difference in OD units between each step of the reference system; d is the OD at 257 mμ of 1 μμg nuclease-digested nucleic acid per square micron. This value is 2.93 for RNA (Edström, 1953) and 2.57 for DNA (Edström and Kawiak, 1961).

4.3. Absorption Integrating Device - The picture of the lens-shaped nucleic acid extracts are considered is consisting of a number of concentric zones and a central circular area, with all zones having the same area as the latter. The average OD of each zone is determined and the values can be added together since they are representations of absorption from areas of equal size. It is possible to calculate the OD from the steps of the reference system. From the value for the magnification and the specific OD constant for the nucleic acid in question, the amount contained in a spot is determined. This procedure is carries out with the aid of an absorption integrating device constructed as follows: A horizontal line, representing background blackening, is drawn on millimeter paper. A vertical line to represent the center of the extract is drawn upwards from the middle of this line. Vertical lines are then placed on both sides of the central line at such distances that they will each divide a zone into areas of the same size. If the central circular area is given a radius of r, the distances of the zone-dividing lines from the center will be $r \cdot \sqrt{0.5}$, $r \cdot \sqrt{1.5}$, $r \cdot \sqrt{2.5}$, etc. The intersection points of the mean curve of the two superimposed photometer curves, drawn on transparent paper, and the zone-dividing lines (the central line should not be included) are registered in millimeters from the base line. The values

on both sides are added, and the sum will constitute an integral of the blackening of the extract. Since it is suitable to divide an extract into about two zones and since the value for each zone represents four points, the integration is based on about forty measuring points. It is practical to have three absorption integrating devices having radii for the central circular area of 0.5, 1, and 1.5 cm, respectively. As the arm magnification of the densitometer can be varied (2,5, or 10 X), it is always possible to find combinations which give a suitable number of measuring points.

F. References

Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955). In "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. I, pp. 493-553. Academic Press, New York.

Beermann, W. (1961). Chromosoma 12, 1.

Brachet, J. (1940). Compt. Read, Soc. Biol. 133, 88.

Davidson, J. N. (1950). "The Biochemistry of the Nucleic Acids," 1st ed. Methuen, London.

de Fonbrune, P. (1949). "Technique de micromanipulation." Monographies de l'Institut Pasteur. Masson, Paris.

Edström, J.-E. (1953). Biochim. Biophys. Acla 12, 361.

Edström, J.-E. (1956). Biochim. Biophys. Acla 22, 378.

Edström, J.-E. (1958). J. Neurochem. 3, 100.

Edström, J.-E. (1960a). J. Biophys. Biochem. Cytol. 8, 39.

Edström, J.-E. (1960b). J. Biophys. Biochem. Cytol. 8, 47.

Edström, J.-E., and Beermann, W. (1962). J. Cell Biol. 14, 371.

Edström, J.-E., and Gall, J. G. (1963). J. Cell Biol. In press.

Edström, J.-E., and Kawiak, J. (1961). J. Biophys. Biochem, Cytol. 9, 619.

Edström, J.-E., and Pigon, A. (1958). J. Neurochem. W, 95.

Edström, J.-E., and Pilhage, L. (1960). J. Biophys. Biochem, Cytol, 8, 44.

Edström, J.-E., Grampp, W., and Schor, N. (1961). J. Biophys. Biochem. Cytol. 11, 549.

Edström, J.-E., Eichner, D., and Edström, A. (1962). Biochim. Biophys. Acta 61, 178.

Egyhazi, E., and Hyden, H. (1961). J. Biophys. Biochem, Cytol. 10, 403.

Gomirato, G., and Hyden, H. (1963). The Brain. In press.

Hartleib, J., Diefenbach, H., and Sandritter, W. (1956). Acta Histocem. 2, 196.

Hyden, H., and Egyhazi, E. (1962). J. Cell Biol. 15, 37.

Hyden, H., and Pigon, A. (1960). J. Neurochem. 6, 57.

Hyden, H., Lovtrup, S., and Pigon, A. (1958). J. Neurochem. 2, 304.

Jaristedt, J. (1962). Exptl. Cell Res. 28, 501.

Kohler, A. (1904). Z. Wiss. Mikroskop. 21, 129.

Lagerstedt, S. (1956). Experientia 12, 425.

Lagerstedt, S. (1957). Z Zellforsch. Mikroskop. Anat. 45, 472.

Markham, R., and Smith, J. D. (1951). Biochem. J. 49, 401.

McFarland, R. H., Anderson, R. A., Nasim, M., and McDonald, D. G. (1958). Rev. Sci. Instr. 29, 738.

Ornstein, L., and Lehrer, G. M. (1960). J. Histochem. Cytochem. 8, 311.

Perry, R. P. Errera, M., Hell, A., and Durwald, H. (1961). J. Biophys. Biochem. Cytol. 11, 1.

Pigon, A., and Edström, J.-E. (1959). Exptl. Cell Res. 16, 648.

Sandritter, W., Pillat, G.,and Theiss, E. (1957). Exptl. Cell Res. Suppl. 4, 64.

Scott, J. F. (1955). In "Physical Techniques in Biological Research" (G. Oster and A. W. Pollister, eds.), Vol. I, pp. 131-203. Academic Press, New York.

Smith, J. D., and Markham, R. (1950). Biochem J. 46, 509.

Stowell, R. E., and Zorzoli, A. (1947). Stain Technol. 22, 51.

Thorell, B. (1947). "Studies on the Formation of Cellular Substances during Blood Cell Formation." Kimpton, London.

Walker, P. M. B. (1955). Exptl. Cell Res. 567.

Walker, P. M. B. (1956). In "Physical Techniques in Biological Research" (G. Oster and A. W. Pollister, eds.), Vol. III, pp. 402-487. Academic Press, New York.

Walker, P. M. B. (1958). In "General Cytochemical Methods" (J. F. Danielli, ed.), Vol. I, pp. 164-217. Academic Press, New York.

Zajieek, J., and Zeuthen, E. (1956). Exptl. Cell Res. 11, 568.