A METHOD FOR DETERMINING TOTAL PROTEIN OF ISOLATED CELLULAR ELEMENTS AND CORRESPONDING TRITIUM RADIOACTIVITY

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ABSTRACT

A method is described for the microanalysis of protein, obtained from isolated tissue elements, in the range of 500 $\mu\mu$g–500 mμg. The method entails solubilization of cellular protein with phosphoric acid and heat after extraction of acid-soluble compounds, lipids, and RNA. A procedure for the extraction and recovery of cellular RNA by the use of 40% trichloroacetic acid is presented. The solubilized protein, in the form of a microdroplet, is photomicrographed with monochromatic light at 230 mμ. Total density in the microdroplet is determined from calibrated photographic plates by microdensitometry, and is converted to protein mass by using an experimentally determined average specific absorbance value. A solubilized protein labeled with tritium can be recovered after photomicrography, combusted, and reduced to generate tritiated gas for high-efficiency tritium radiometry. Total protein was analyzed in (*a*) nerve cells of three different sizes from Deiters' nucleus of the rabbit; and the whole rod cell and rod cell nucleus of the rabbit retina.

INTRODUCTION

Analytical chemistry of single cells is a goal often sought but difficult to achieve. To the biologist, nucleic acids and proteins represent two important classes of cellular constituents of major interest. Yet, only in the case of nucleic acids are methods (Edström, 1958, 1960) available for the analysis of isolated cells and dissected cellular components which satisfy the basic methodological criteria of sensitivity, specificity, and reliability. In the case of proteins, quantitative methods of comparable applicability have not been developed.

An ultramicro method is extended in usefulness considerably when it can be coupled with quantitative radiometry. The tritium isotope is well suited for studies at the cellular level because labeled precursors of proteins and nucleic acids can be obtained with high specific radioactivity. However, the absorption of the low-energy beta radiation poses a serious impediment to achieving high efficiency for tritium detection. A microcombustion procedure (Koenig and Brattgård, 1963) was developed for the Edström microelectrophoretic separation of pyrimidine nucleotides and purine bases (Edström, 1960), in line with such reasoning.

This report describes a method for quantitatively analyzing the total protein, of isolated cells and parts of cells, in the range of about 5×10^{-10}–5×10^{-7} g. In principle and technique, the method is based on that of Edström for the analysis of total cellular RNA (Edström, 1958). It entails integrating the total density of microdroplet samples of cellular protein solubilizates from calibrated photomicrographs taken with monochromatic light. In addition, tritium radioactivity in protein or RNA microdroplet samples can be determined with high efficiency by an adaptation of the microcombustion procedure (Koenig and Brattgård, 1963); this method has been employed to study in

vitro incorporation of tritiated precursors into axonal protein and RNA (Koenig, 1967 *a*, 1968).

Edström's review (1964 *a*), which is a comprehensive treatment of his methods and techniques for the analysis of RNA from isolated tissue units, is recommended as an important adjunct to the analytical procedure described below for protein. Prerequisite instrumental and technical information not presented here is included in that reference.

PREPARATIVE PROCEDURES

Preparation and Preliminary Extraction

Cells or parts of cells may be isolated by any convenient means, i.e. free hand dissection under a stereomicroscope such as that used by Hydén (1959), or microdissection of deparaffinized sections of fixed tissue under phase-contrast microscopy (Edström, 1964 *a*). Once isolated, the tissue elements are transferred to a miniature droplet of an isotonic solution or of distilled water on a previously halved coverslip. The cover slip must be long enough to span the well of an oil chamber (see Edström, 1964 *a*).

The tissue elements are air dried on the cover slip which then is passed through the following solutions, successively: (*a*) 10% trichloroacetic acid, 5 min; (*b*) absolute ethanol, 5 min; and (*c*) chloroform-methanol (2:1, v/v), 5 min.

RNA Extraction

The extraction of RNA from isolated tissue elements can be performed by enzymatic or nonenzymatic means. The enzymatic digestion with ribonuclease (RNase) is done according to the Edström procedure: incubation at 37°C for 1.5 hr with 0.4 mg/ml RNase (Worthington Biochemical Corp., Freehold, N. J.) and 0.02 M ammonium acetate buffer, adjusted to pH 7.3 with NH_4OH.

A nonenzymatic extraction procedure has been worked out as an alternative; it involves a partial hydrolysis of cellular RNA to oligonucleotides by 40% trichloroacetic acid at 37°C for 1.5 hr. No further RNA can be recovered with RNase after partial trichloroacetic acid hydrolysis. Electrophoresis of trichloroacetic acid–extracted RNA from nerve cells of Deiters' nucleus, moreover, shows no contamination by protein, but the base composition o this RNA differs somewhat from that of RNase digests reported (Hydén and Egyházi, 1962) for the same cell type (Table I). There is evidence, through the use of incorporated radioactive RNA precursors (Koenig, 1967 *a*, 1968), that RNase digestion does not remove all of the RNA from isolated, myelin-free axons. The RNA that is spared in the axon is significant and appears to be a stabilized fraction which is associated with the axolemma. It is, however, quite resistant also to mild acid treatment (1 N HCl at 37°C for 24 hr) (Koenig, E. 1968. Unpublished data.).

TABLE I

The Base Composition of RNA of Nerve Cells from Deiters' Nucleus of Rabbit Extracted by the Trichloroacetic Acid Procedure

Base	No. determinations	Mode of RNA Extraction*	
		40% trichloroacetic acid	Ribonuclease digestion‡
Adenine	22	18.60 ± 0.64	19.7 ± 0.37
Guanine	22	30.14 ± 0.60	33.5 ± 0.37
Cytosine	22	29.23 ± 0.73	28.8 ± 0.36
Uracil	22	21.97 ± 0.63	18.0 ± 0.18

* Molar proportions in per cent of sum (±SE).
‡ From Hydén and Egyházi, 1962.

If the trichloroacetic acid–extracted RNA is to be analyzed for total amount of RNA or for base composition according to the Edström procedure (Edström, 1964 *a*), then the trichloroacetic acid is removed, and the RNA is recovered quantitatively as follows. The extract is recovered from the tissue element after incubation and is transferred to a medium of triacetin-dimethylphthalate (T-DMP) or ethylene glycol monomethyl ether–dimethylphthalate (1:10 v/v). The trichloroacetic acid hydrolysate is deposited on the underside of a cover slip (*2* in Fig. 1) which lies in a T-DMP medium on an oil chamber; immediately in front of this cover slip is a cover slip (*1* in Fig. 1) the underside of which contains the cellular residue in paraffin oil (Fig. 1). An air space of 2 mm or more separates the oil from the T-DMP

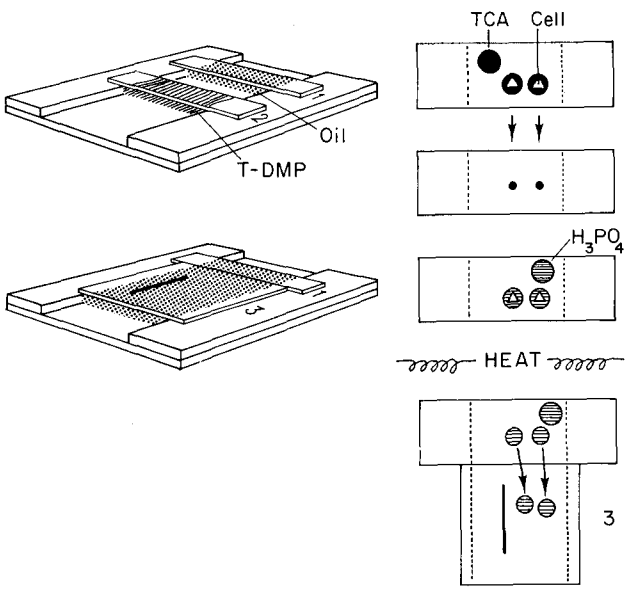

FIGURE 1 A graphic, schematized summary of the procedure for extraction of RNA by 40% trichloroacetic acid (*TCA*), recovery of RNA, solubilization of cells, and transfer of solubilized protein to the quartz glass slide. Steps are as follows: (*a*) incubate cells with 40% trichloroacetic acid in oil chamber at 37°C for 1.5 hr (*1*, upper right); (*b*) transfer RNA extracts to T-DMP (*1*, *2*, upper left and right); (*c*) apply 25% H_3PO_4 to cells (*1*, middle right); (*d*) heat (110–115°C) for 5 min; and (*e*) place quartz glass slide on oil-chamber and transfer-cell solubilized proteins (*1*, *3*, lower left and right). See text for details. *1*, cover slip with adhering cells; *2*, cover slip with trichloroacetic acid extracts, i.e., oligonucleotides; *3*, quartz glass with cell solubilized protein.

in the oil-chamber well. When the trichloroacetic acid hydrolysate is expelled, the aqueous solvent phase and trichloroacetic acid diffuse into the T-DMP medium and thus leave behind on the cover slip a dehydrated oligonucleotide residue. The rate of diffusion of trichloroacetic acid from the extract into the T-DMP can be accelerated by increasing the T-DMP ratio. The cover slip (*2*, Fig. 1) with the dried RNA is removed from the oil chamber and washed with petroleum ether and chloroform for removal of the adhering layer of oil. The cover slip is placed on another, unused oil chamber, whereupon the extract can be dissolved with ammonium-acetate buffer (0.02 M) for transfer to a quartz glass slide for determination of total RNA, or the extract can be dissolved with 4 N HCl for complete acid hydrolysis and electrophoresis (see Edström, 1964 *a*). In the latter instance, however, hydrolysis should be carried out for only 18 min instead of 30 min as is the case for RNase digests.

Solubilization of Cellular Protein Residue

After RNA extraction, the cover slip (*1*, Fig. 1) with the cellular residue is removed from the oil chamber and washed first with petroleum ether, and then with $CHCl_3$ to remove the paraffin oil. The cover slip is returned to the oil chamber, and the chamber well is filled with Dow Corning Corp., (Midland, Mich.) silicon oil (350 C.P.), saturated with 25% H_3PO_4. Silicon oil is preferred over paraffin oil, for two reasons: (*a*) it absorbs less radiation in the far ultraviolet, and (*b*) it allows diffusion of water more readily, and thus permits a concentration of the H_3PO_4 which promotes solubilization (see below).

A small depot droplet of 25% H_3PO_4 is placed on the underside of the cover slip. A volume sufficient to envelop the tissue element is transferred from the depot droplet to each element in turn (see Fig. 1) with a micropipette. The oil chamber is racked out of the optical path, and is brought into proximity with a heater mounted behind the objective nosepiece of the microscope (see Edström, 1964 *a*). The heater is turned on. As measured with a thermocouple in the oil at the cover slip undersurface, a temperature of 110°–115°C is sufficient to solubilize the cellular protein completely in about 5 min (Fig. 2). Excessive temperature, with long exposure time, should be avoided because it produces an increase in absorption in the ultraviolet that appears to be related to tryptophan content (see Results).

A line is drawn, with water-soluble ink, on the surface of a clean, dry quartz glass, 24 × 30 × 0.5 mm. The quartz glass is inverted (ink side down) and placed behind and in contact with the cover slip containing the solubilized material. The well of the oil chamber, underlying the quartz glass, is filled with 25% H_3PO_4-saturated silicon oil. Approximately an additional half volume of 25% H_3PO_4 from the depot droplet is applied to each solubilized sample to lower the protein concentration. The refractive index changes with dilution, and the brightness contrast of the solubilized material is lost but returns again slightly as the final portion of H_3PO_4 is added. The diluted solubilized material is recovered in a micropipette and transferred to a suitable location

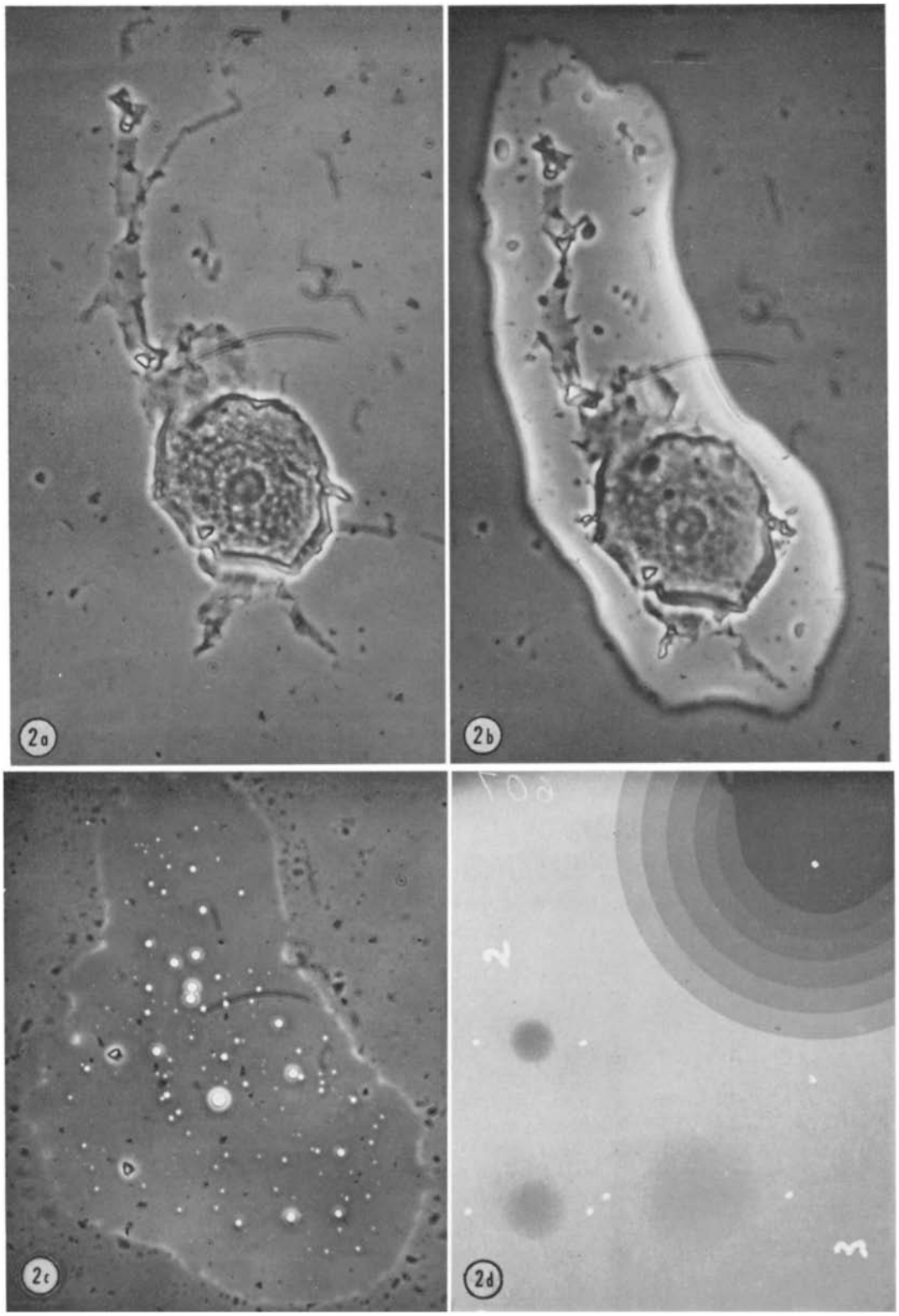

FIGURE 2 *a*, A nerve cell after it has been taken through the preliminary extraction procedure. *b*, The application of 25% phosphoric acid to the nerve cell. *c*, The solubilization of the cell after 5 min of heat (110°–115°C). *d*, A photomicrograph of solubilized proteins of three nerve cells taken at 230 mμ; the dots indicate the scanning path to be taken by the microdensitometer. Fig. 2 *a*, *b*, and *c*, × 310; Fig. 2*d*, × 80.

near the ink line on the quartz glass where it is expelled to form a microdroplet (*3*, Fig. 1). When all of the solubilized samples have been transferred, the quartz glass is removed from the oil chamber, turned upright (ink side up), and placed with the adhering oil layer in a holder (see Edström, 1964 *a*).

Ultraviolet Photomicrography of Solubilizate

The instrumentation in use in this laboratory for monochromatic ultraviolet photomicrography includes the following: (*a*) a Cooke, Troughton and Simms (now Vickers, York, England) ultraviolet microscope; (*b*) a Bausch & Lomb Incorporated (Rochester, N. Y.) grating monochromator (500 mm focal length; diffraction grating, 1200 lines per millimeter) equipped with a condenser for microscopy; (*c*) an Hanovia 200 W DC xenon-mercury compact arc lamp and power supply (Engelhard Hanovia Inc., Newark, N. J.). The microscope is equipped with a visual tube that contains a 10× fluorescent eyepiece, a 16 mm monochromat quartz objective, a 5× quartz ocular, a quartz condenser with the front two lenses removed to increase the field of illumination, and a camera that accommodates 3¼ × 4¼-inches photographic glass plates (Kodak metalographic plates). Mounted in the corner of the camera under the photographic plate is a rotating sectored disc designed to generate five isodensity zones with constant increments of density of 0.1505 OD units between adjacent zones. The isodensity zones provide an internal standard for calibrating the density of the photographic plate. Edström's papers (1958; 1964 *a*) should be consulted for details relating to the design of the sectored disc.

The soluble samples are photographed with the rotating, sectored disc at 230 mμ (total band width, 3.3 mμ) (Fig. 2). The substage condenser is adjusted for Köhler illumination (the field stop is imaged in the object plane). The inked line on the quartz glass is an easy, opaque landmark to locate, and its border can be used for critical focusing. If the solubilized samples are labeled with tritium, the samples can be recovered for combustion radiometry after photomicrography (see below).

Determination of Total Protein in a Sample Solubilized Protein

Total protein is computed from density curves, obtained with a Joyce-Loebl microdensitometer, after scanning photomicrographic sample spots and calibration isodensity zones, a method similar to that for RNA analysis as done by Edström (1958, 1964 *a*). However, the integration of total density of the microdroplet is arrived at mathematically, by using as data the area under the density curve, the radius of the density-curve base line, and the average height between adjacent isodensity zones. The area under the density curve is determined by tracing the curve on tracing paper, cutting it out, and weighing it to the nearest microgram.

The theoretical rationale for using the mathematical integration procedure is based on the theorem of Pappas which states that the volume generated by revolving a plane area about its coplanar axis, fixed in space, is equal to the product of the plane area and the circumference of the circle described by its centroid. The centroid can be conceived as a line parallel to the fixed, coplanar axis, along which the area's center of gravity is located. Thus, $V = 2\pi \bar{x} A$; where V is the volume, $\bar{x}$ is its centroid, and A is the plane area. Since the density curve of a sample spot is parabolic, it can be shown that the distance between the fixed axis and the centroid of a parabolic plane figure is given by $\frac{3}{8} b$; where b represents the radial distance of the base line between the fixed axis and the lateral limiting boundary of the area (Fig. 3). Since the area under the density curve is symmetrical about a central axis, it suffices to rotate the area 1π about its central axis in order to generate the volume of density. The volume of density, standardized and corrected for magnification, divided by the specific absorptivity coefficient of protein at the wavelength photographed will yield total protein. The expression derived to compute total protein is as follows:

$$\text{micromicrogram protein} = \frac{\pi \frac{3}{8} bA\ (10{,}000/m)^2\ 0.1505}{0.380\ \bar{h}};$$

where, b is ½ the distance along the base line of the density area in centimeters, A is area under the density curve in square centimeters, m is total magnification (microscopic × densitometric), 10000 is conversion of centimeters to microns, 0.1505 is difference in optical density between adjacent calibration isodensity zones, $\bar{h}$ is the average height in centimeters of the set of 0.1505 density calibration steps, and 0.380 is absorbance of 1 μμg protein/μ^2 in H_3PO_4 at 230 mμ.

Tritium Radiometry of Solubilized Protein

The advantage of using tritium-labeled precursors, particularly for ultramicroanalytical work, is the high specific radioactivity that can be obtained. A serious impediment, however, is tritium's low energy of emission, and, therefore, low detection efficiency. This difficulty can be surmounted in large part by combusting the tritium-labeled organic material and by generating tritiated gas by reduction. Of course, high efficiency alone is not enough, but must be coupled with low background counting so that the relative error of measuring low absolute levels of radioactiv-

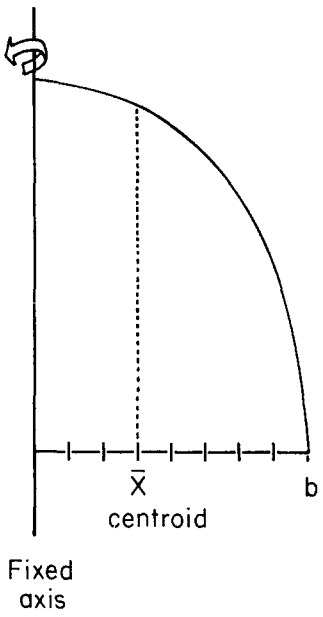

FIGURE 3 A parabolically shaped, plane area, characteristic of one-half of a density-curve tracing, that is to be rotated through 2π.

ity can be reduced. A method utilizing this approach was developed for analyzing tritiated RNA hydrolysates extracted from groups of isolated cells and separated by microelectrophoresis (Koenig and Brattgård, 1963). An adaptation of the combustion procedure for soluble proteins is given below.

After photomicrography, the quartz glass with solubilized protein samples (RNA droplets may be handled the same way) is inverted over an oil chamber and the chamber space is filled with silicon oil. A depot droplet of 25% H_3PO_4 is placed on the underside of the quartz glass. A volume of 25% H_3PO_4 is taken up in a new micropipette without the usual preliminary step of drawing up a column of oil. The phosphoric acid is expelled in a solubilized sample, by diluting it about 1.5–2.0-fold (the amount of dilution is not critical). The diluted sample is taken up quantitatively in the new micropipette, and a short column of oil is drawn in behind it to fill the tip. Although not essential, it is helpful to rinse off, with petroleum ether, the oil adhering to the outside surface of the micropipette. The micropipette is mounted by two alligator clips in a special holder often used by watchmakers for soldering (soldering jig), and the micropipette tip is centered in the field of a stereomicroscope, about 1 inch above the base. A magnification of about 6 is helpful for breaking off the tip and inserting it into a capillary bomb.

A Pyrex capillary (0.5 mm, I.D.) is sealed in a microflame at one end and separated from the rest of the tubing about 2 cm from the closed end. A mixture of finely powdered $KClO_4$ and zinc (1:1 w/w) is layered in the bottom of the sealed end for a depth of about 2 mm, and the capillary is temporarily laid aside. The shank of the micropipette is grasped just beyond the meniscus of the fluid column and held firmly, but without excessive pressure, by the serrated tips of a fine dissecting forceps (forceps must be flexible) in the preferred hand. With the other hand, a small wire nipper is applied behind the forceps, on the proximal side of the micropipette, i.e., away from the tip. The nipper is used to crush the shank at the point of application; this spares the distal portion of the shank and tip held by the forceps.

The micropipette tip is inserted into the capillary and allowed to fall to the $KClO_4$-zinc mixture at the bottom. A syringe-needle stylet can be used as a ramming rod to drive the tip into the mixture at the bottom. Another 2–3 mm of the $KClO_4$-zinc mixture is added and tapped down by holding the capillary bomb between the thumb and forefinger and tapping on the table top. An additional 5–6 mm of finely powdered zinc alone is added and tapped down. The open end of the capillary is sealed in the following manner. The portion of the sealed end of the capillary is held rigidly in the tips of a coarse tissue forceps over a microflame. The flame is applied just beyond the end of the column of zinc; the open end of the capillary is grasped with the flexible fine dissecting forceps, and the unfilled end of the capillary is separated from the filled closed end by pulling the glass out after it has softened. The drawn out end is inserted in the flame and sealed; the over-all length is 1.0–1.5 cm.

About three-fourths of the lower portion of the capillary bomb ($KClO_4$-zinc end) is inserted into the center space of a spiral wire heating element, and the current is turned on. The heater in use in this laboratory reaches 580°C in the center space within 1 min and levels off at 610°C; the capillary bomb is heated at this temperature for 4 min. The capillary bomb is then removed and inserted into a specially designed Geiger-Mueller tube (G.-M.) (Koenig and Brattgård, 1963). Geiger gas is flushed through and the G.-M. tube is closed; the bomb is crushed in the closed tube, and the tube is placed in a steel castle under an umbrella of 13 industrial G.-M. tubes (1 ft. in length). Disintegrations are counted in an anticoincidence mode. In a slightly modified G.-M. tube presently being used, efficiency for tritium detection is approximately 80% and the background is less than 1 cpm.

RESULTS AND DISCUSSION

The Choice of Wavelength for Measurement

Proteins and polypeptides absorb at an increasing rate below 250 mμ. A wavelength of 230 mμ

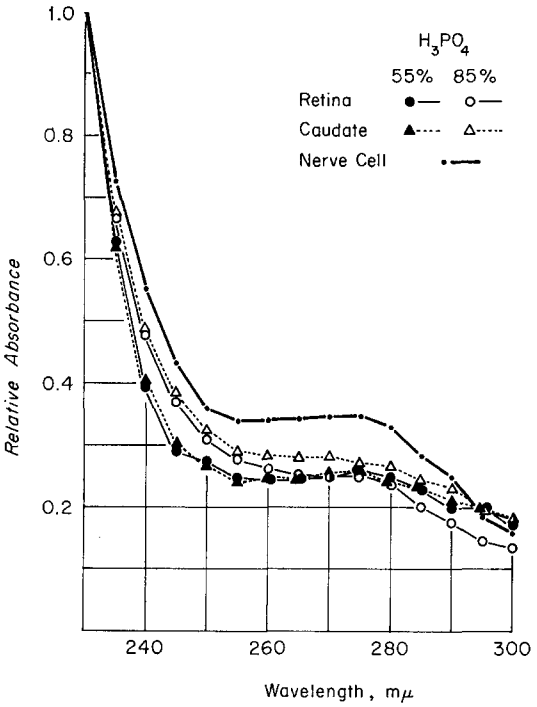

FIGURE 4 The relative ultraviolet absorbance of protein solubilized with either 55 or 85% phosphoric acid and heat (115°C; 55%, 30 min; 85%, 15 min). The nerve cell was solubilized by the procedure under Preparative Procedures.

was selected for protein determination, for several reasons, notwithstanding the fact that it is not an absorption peak: (*a*) the nucleic acid absorption curve goes through a minimum at this wavelength; (*b*) radiation intensity diminishes beyond practical microscopic visualization with shorter wavelengths; and (*c*) photographic plate emulsion is too insensitive for shorter wavelengths.

Individual purified proteins, e.g. serum protein fractions, exhibit different specific absorbancies at 230 mμ. For this reason an average representative absorbance value was determined for crude tissue protein-extracts. The retina and caudate nucleus from rabbit were used for this purpose, because they represent tissues high in cell density and lack connective tissue investments. Extraction was carried out as follows. Retina or caudate nucleus was homogenized in a solution of 0.02 M ammonium acetate and 0.01 M $MgCl_2$ (pH 7.3) containing 0.4 mg/ml each of RNase and DNase, and incubated for 2 hr at 37°C with shaking. Trichloroacetic acid was added to a final concentration of 10% at the end of incubation. The precipitate was sedimented and washed successively with 10% trichloroacetic acid (three times), absolute ethanol (twice), chloroform-methanol (2:1, v/v; three times), and acetone (three times). The average specific absorbance at 230 mμ of 0.1 mg/ml retinal protein in 55% H_3PO_4 was 0.382, while that of 0.1 mg/ml caudate nucleus protein in 55% H_3PO_4 was 0.377; this yielded a mean specific absorbance of 0.380.

The crude protein extract does not dissolve in cold concentrated phosphoric acid, but will dissolve with heating when the concentration of phosphoric acid is 55% or greater; this is true also for most purified proteins tried, with the exception of trypsin and ribonuclease which are soluble in cold H_3PO_4. An ultraviolet absorption spectrum of protein from caudate nucleus and from retina in 55 and 85% H_3PO_4 is shown in Fig. 4. Typically, the absorption peak between 270 and 280 mμ, due to the aromatic amino acid residues (mainly tryptophan and tyrosine), is markedly diminished under strongly acidic conditions (Fig. 4). Prolonged heating at 110–115°C causes an increase in absorption at 230 mμ of most proteins dissolved in concentrated H_3PO_4 (Fig. 5 *b*). Since crude protein is not soluble in cold H_3PO_4, the early effects of heat on absorptivity (<15 min) were examined in the case of trypsin and RNase, which are soluble. An exposure to 115°C for up to 12 min produces no appreciable change in absorption (Fig. 5 *a*). However, longer exposure to this temperature results in an increasing absorptivity only in the case of trypsin, not in the case of RNase. The reason for the disparate behavior of these two proteins may be associated, at least in part, with the relative content of tryptophan. RNase has no tryptophan, but trypsin has four tryptophan residues (see Mahler and Cordes, 1966). Moreover, heat (115°C) applied for 30 min to tryptophan alone in concentrated phosphoric acid increases its absorbance at 230 mμ by about 50%; in addition, the absorption spectrum of tryptophan between 230- and 300-mμ changes in a very striking fashion, besides showing an increased absorptivity throughout this region (Fig. 6). It seems likely, therefore, that for most proteins both the flattening of the absorption curve between 250 and 280 mμ with strong acid and the increase in magnitude of absorbance with heating may be attributed largely to actions upon tryptophan residues.

The stability of absorbance was tested on crude retinal protein and caudate nucleus protein in

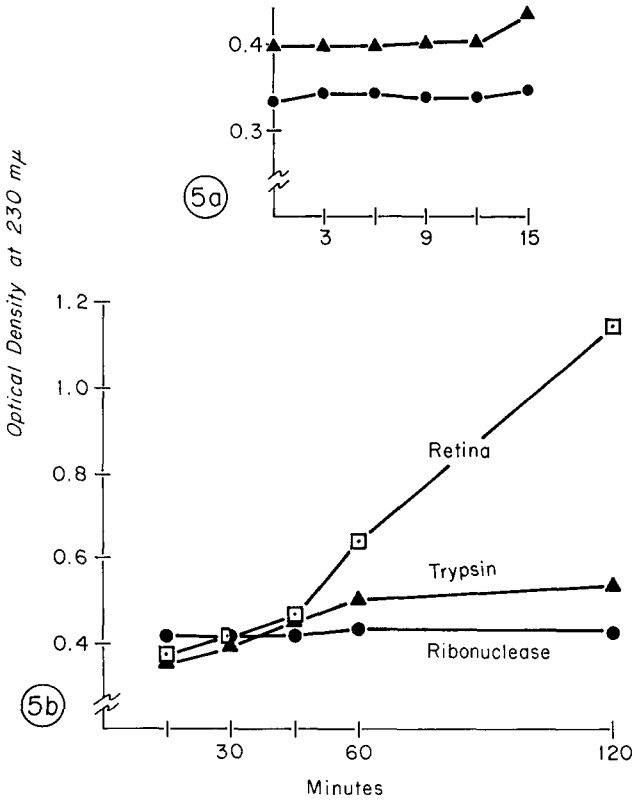

FIGURE 5 *a*, The early effects of heat (115°C) on ultraviolet absorbance of ribonuclease and trypsin, which are soluble in cold 85% phosphoric acid. *b*, Effects of prolonged heat (115°C) on ultraviolet absorbance of protein, i.e. ribonuclease, trypsin, and crude retinal protein, dissolved in 85% phosphoric acid.

concentrated phosphoric acid solution. A loss of absorbance with time was observed. After the proteins had been standing at room temperature for 3 days in 55% H_3PO_4, absorbance at 230 mμ for retinal protein fell to 92% of initial absorbance, and for caudate nucleus protein to 88% of initial absorbance; after 8 days, absorbance for retinal protein had fallen to 82% of initial absorbance. However, absorbance at 230 mμ was least affected compared to that at longer wavelengths; the fall in absorbance for retinal protein for the whole range of 230–300 mμ was 86% after 3 days, and 73% after 8 days; caudate nucleus protein showed an average fall in absorbance of 73% of initial absorbance after 3 days. These data would suggest that the chromophore(s) absorbing in the region above 230 mμ is more greatly affected upon standing; this again implicates tryptophan which is known to be destroyed during hydrolysis of protein with strong acid (see Canfield and Anfinsen, 1963). No doubt some of the loss of absorbance upon standing may be traced to some protein degradation, in addition. Finally, there would seem to be also a greater absorbance lability of caudate nucleus protein compared with retinal protein.

An absorption spectrum of a protein solubilizate from an isolated nerve cell is similar to that of crude protein extracts (Fig. 4). Relative absorbance was determined by direct microspectrophotometry, with a photomultiplier tube mounted atop the ultraviolet microscope. Extinction values were read from a Schoeffel Instruments photometer (Westwood, N.J.).

A serial dilution of retinal protein, with the photomicrographic-densitometric procedure, is shown in Fig. 7. The volumes of the samples were kept constant, and were transferred with a calibrated micropipette (see Edström, 1964 *b*). The amounts transferred ranged from 0.64 to 96 mμg; all amounts recovered were generally within 10% of the theoretical amount applied. The lower limit of sensitivity is of the order of 500 μμg; the upper practical limit is probably 500 mμg.

Sources of Error

In light of what has been considered above, three major potential errors should be discussed. One of

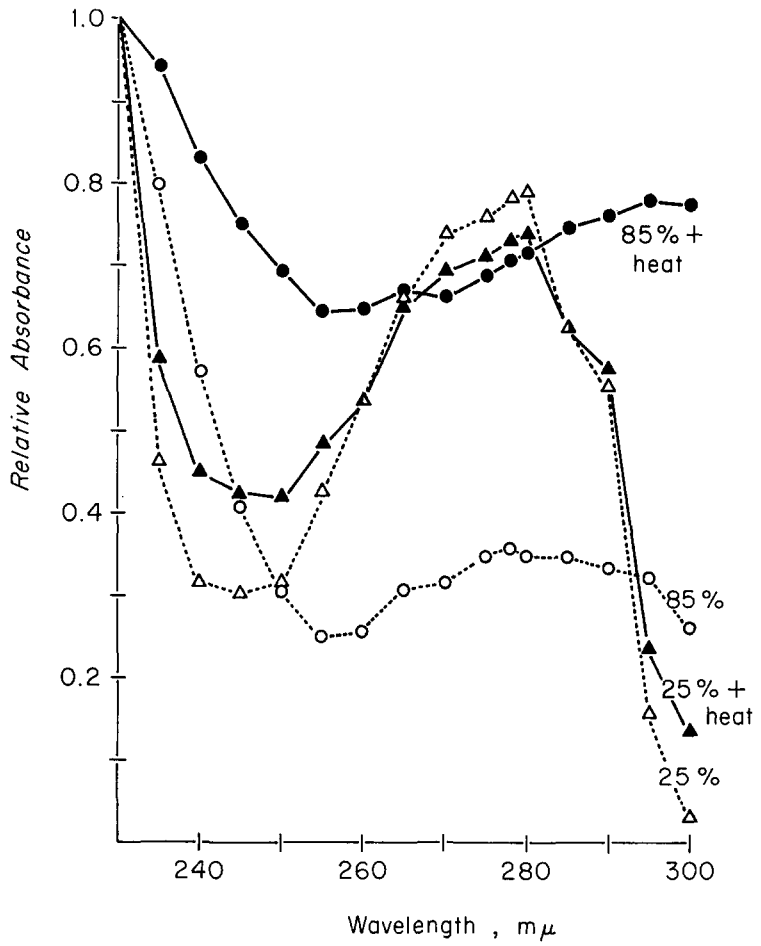

FIGURE 6 Effects of heat (115°C, 30 min) on ultraviolet absorbance of tryptophan dissolved in 25 or 85% phosphoric acid.

them is relatively insignificant in most cases (an exception is considered below), but the other two must be guarded against. The first possible error relates to the incomplete extraction of nucleic acids. DNA can be removed by preincubation with DNase; however, the DNA content of a single cell is small (about 7 μμg) and would not contribute measurably to absorption at 230 mμ; nor would any residual RNA that resists RNase digestion or mild trichloroacetic acid hydrolysis (see Preparative Procedures). A second possible source of error may arise with excessive heating during solubilization. This error can be avoided if exposure to heat is kept to a minimum, i.e., <10 min at 110–115°C. The third and probably the largest error that could be introduced would be the preparation of a solubilized protein that is too concentrated; this would result in a density that exceeds the sensitivity of the photographic emulsion (zero transmission). Under such circumstanes, the quartz glass would have to be returned to the oil chamber, the sample would have to be diluted with 25% H_3PO_4, and the diluted sample would have to be relocated to another position on the quartz glass.

Values Obtained for Isolated Cellular Elements

Determination of total, insoluble protein was conducted on moderately large, large, and very large nerve cells of Deiters' nucleus from rabbit that were isolated by free hand dissection in the following medium: 0.05 M aminomethane sulfonic acid, 0.05 M sodium acetate, 0.05 M diethylamine

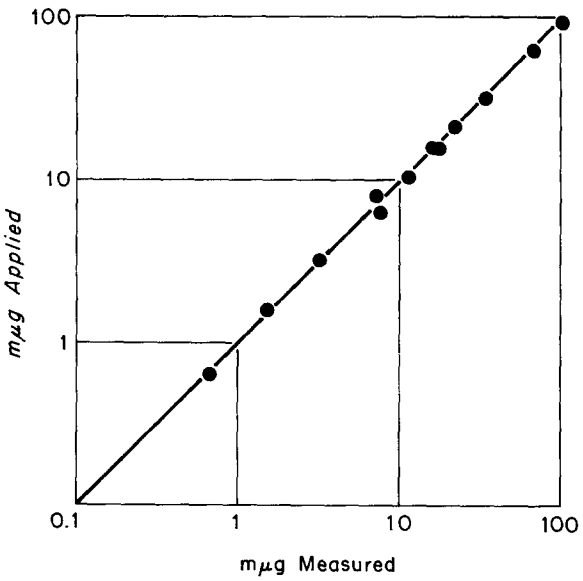

FIGURE 7 A dilution series of a stock retinal protein solution of known concentration, determined according to the photomicrographic-densitometric procedure.

TABLE II

Total Protein of Nerve Cells from Deiters' Nucleus of Rabbit Compared According to Procedure for RNA Extraction

	Mode of RNA Extraction			
	Ribonuclease		40% trichloroacetic acid	
Relative size of cells	Protein	No. determinations	Protein	No. determinations
	mμg		*mμg*	
Moderately large	11.38 ± 1.31	8	11.68 ± 1.24	11
Large	17.21 ± 2.19	11	16.55 ± 1.87	21
Very large	26.34 ± 3.25	11	23.49 ± 2.25	4

HCl, and 0.05 M Tricine (*N*-methyl-tris-hydroxymethyl-glycine), and 0.01 M dithiothreitol (Cleland's reagent). A comparison was made of the three groups of cells in terms of the total non-lipoprotein protein values obtained according to whether RNase or 40% trichloroacetic acid was used for RNA extraction. The results are shown in Table II. The protein mass for nerve cells from Deiters' nucleus of the rabbit has been reported to be 16.6 mμg (Hydén, 1959), based on a method of soft X-ray absorption radiography. This value compares favorably with the values obtained for the large nerve cells.

Another series of protein determinations were carried out on rod cells and rod cell nuclei of the rabbit retina. Single rod cells and denuded rod cell nuclei were isolated by microdissection (see Koenig, 1967 *a*, for technique). Each sample consisted of 200–400 rod cells or rod cell nuclei. This cell type is instructive insofar as it illustrates a case in which unextracted DNA can introduce a very significant error, and in which the trichloroacetic acid procedure for extracting RNA can not be employed.

The total RNA content of a rabbit rod cell is only 0.65 μμg, 40% of which is nuclear (Koenig, 1967 *a*). The DNA content, therefore, is an order of magnitude greater, i.e., 7 μμg per cell (see Edström and Kawiak, 1961). Since at least 200 cells are required for a single sample analysis, the error would be appreciable if DNA were not removed before solubilization. Partial hydrolysis by 40% trichloroacetic acid can not be used because it

solubilizes the cell at room temperature. The latter observation is consistent with earlier findings regarding the peculiar properties of the plasma membrane that distinguish the rod cell from nerve cells in general (see Koenig, 1967 *b*).

The rod cells or rod cell nuclei were preincubated with RNase and DNase (0.4 mg/ml each) for 2 hr at 37°C before solubilizing with phosphoric acid. The results are given in Table III. Those lipoproteins which would have been extracted by the chloroform-methanol after 5% trichloroacetic acid (see Methods) are probably not significant. The values obtained, however, may be low from the standpoint of total cell protein.

TABLE III

Total Protein of the Rod Cell and the Rod Cell Nucleus of the Rabbit

Whole rod cell	No. determinations	Rod cell nucleus	No determinations	Nucleus/whole cell (w/w)
μμg protein		*μμg protein*		%
19.0 ± 1.0	8	9.6 ± 0.9	9	50

Radiometric Determination of Axonal Protein

The method, including the radiometric adaptation, has been applied successfully to the problem of in vitro protein biosynthesis in the axon (Koenig, 1967 *a*, 1968). In these studies, the intracranial portion of the spinal accessory nerve of the rabbit was incubated at 35°C for 5 hr in a synthetic medium (Koenig, 1967 *a*) containing nominally 500 μc/ml of leucine-^{3}H. An experiment is shown in Table IV to illustrate the type of results that were obtained. Denuded, myelin-free axons were isolated and collected after incubation (Koenig, 1965). A medium different from that used earlier (Koenig, 1967 *a*) is now being used to exteriorize the axon: 0.05 M ethylenediamine, 0.05 M citric acid, 0.05 M aminomethane sulfonic acid, 0.10 M glycine, 0.05 M diethylamine HCl, 0.025 M $AlNH_4(SO_4)_2 \cdot 12H_2O$ and 0.001 M H_5IO_6. The axons are collected in clumps of about 80–100 mμg protein (about 7–10 cm axon). Analysis of total axonal protein and corresponding radioactivity was carried out according to the procedure described under Preparative Procedures. Notwithstanding the amount of protein of the axonal samples being generally 5–20 times that of a single cell, it seems likely that rates of incorporation of radioactive label into the nerve cell probably would be higher and would compensate to the extent that levels of radioactivity would be adequate for measurement. In any event, several cells can be pooled. Thus, the method provides a quantitative analytical procedure for studying protein synthesis in specific cell types at a cellular level without resorting to radioautography.

TABLE IV

*Incorporation of Leucine-^{3}H into Axonal Protein In Vitro**

Axonal sample	Total protein	cpm	Specific activity
	μg		*cpm/μg*
1	0.090	21.2	236
2	0.079	20.9	265
3	0.068	48.1	707
4	0.083	43.9	529
5	0.041	49.7	1211
6	0.030	9.1	302

* Incubated for 5 hr with nominally 500 μc/ml leucine-^{3}H at 35°C.

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