Fractionation of Brain Macromolecules

CHROMATOGRAPHY ON HYDROXYAPATITE OF SOLUBLE PROTEINS AND NUCLEIC ACIDS FROM WHOLE RAT BRAIN AND THE EFFECT OF AUTOLYSIS

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(Received 22 March 1965)

1. A procedure for the chromatographic fractionation of soluble brain proteins on calcium hydroxyapatite is described. Chromatograms obtained are reproducible; approximately 11 protein and at least three nucleic acid components can be identified. The effects of column dimensions and flow rate on the chromatograms obtained are described. 2. One of the nucleic acid components appears to correspond to soluble RNA and another to ribosomal RNA. Treatment of homogenates by procedures that cause the removal of ribosomes from soluble extracts also cause the disappearance of the component corresponding to ribosomal RNA. Centrifugation of rat-brain homogenates prepared in 0.25M-sucrose at 105400g for 90min. causes the sedimentation of ribosomes as well as the disappearance of the component corresponding to ribosomal RNA. Extraction of brain tissue, or particulate fractions prepared from brain, with dilute buffers causes the solubilization of part of the ribosomal RNA that then can subsequently be identified in the chromatogram. 3. Autolysis under aerobic conditions has been shown to cause an increase in the nucleic acid component in the chromatogram that corresponds to the ribosomal RNA. Aerobic autolysis causes part of the ribosomal RNA to be solubilized, as evidenced by its failure to be sedimented by centrifugation at 105400g for 90min. and by its appearance in the fraction corresponding to ribosomal RNA in the chromatogram. These changes were not observed when autolysis was carried out under anaerobic conditions. Aerobic autolysis also caused changes in those proteins that are strongly adsorbed on the gel. 4. In general, the proteins of the cell sap appeared to be fairly stable to autolysis. Marked differences in the chromatograms are observed when soluble proteins from the particle fraction were autolysed and chromatographed.

The identification of individual proteins in soluble extracts prepared from brain tissue has attracted the attention of many workers. Almost all of this work has been concerned with the use of electrophoretic techniques. Examination of electrophoretograms permits the qualitative and quantitative estimation of individual protein species as well as the detection of changes in these values due to pathological, pharmacological or physiological stresses. These methods, unfortunately, do not permit the easy isolation of sufficient quantities of material to allow for further purification and characterization. Several workers have only recently turned their attention to developing preparative procedures that would serve to accomplish this purpose. Bogoch, Rajam & Belval (1964) have reported the separation of large quantities of protein from human grey matter by using DEAE-

cellulose chromatography. Bondy & Perry (1963) used DEAE-cellulose to fractionate brain proteins in order to study the incorporation of isotope into individual fractions. Brunngraber & Aguilar (1962) have used calcium hydroxyapatite to fractionate proteins that had been solubilized with the non-ionic detergent, Triton X-100.

The present work describes a simple procedure for fractionating brain proteins and nucleic acids by using calcium hydroxyapatite as the adsorbent in column chromatography. This material was found to give reproducible chromatograms. Large amounts of protein can be processed with little difficulty to permit the isolation of sufficient material for further fractionation by other techniques. The use of this method to study changes during autolysis of homogenates prepared from brain tissue is also described.

METHODS

Column chromatography and preparation of tissue extracts. Calcium hydroxyapatite was prepared by the method of Tiselius, Hjerten & Levin (1956). The gel was allowed to settle by gravity to a depth of 20 cm. in a column of inside diameter 1.3 cm. and fitted with a sintered-glass filter at the bottom. The gel, which is stored in the presence of 1 mM-potassium phosphate buffer, pH 6.8, was equilibrated for at least 2 hr. by running through a solution of this buffer by gravity. The rats used were males of the Wistar strain obtained from the Holtzmann Co., Madison, Wis., U.S.A. Soluble proteins from whole brains were prepared by centrifugation of homogenates (one rat brain/10 ml. of 0.25 M-sucrose) at high speed (105 400g for 30 min.). Loose-fitting TenBroeck homogenizers were used. An amount of extract containing approx. 60 mg. of protein, as determined by the biuret reaction (Mokrasch & McGilvery, 1956) with bovine serum albumin as standard, was allowed to soak into the hydroxyapatite. After the proteins had entered the column, approx. 80 ml. of starting buffer was used to wash the column in order to remove unadsorbed proteins. The remaining proteins were eluted with a concave potassium phosphate gradient. Fractions (5 ml.) were collected in a volumetric fraction collector. The chromatographic development was generally complete in 24 hr. The gradient was achieved by using the Varigrad device, described by Peterson & Sober (1959). The limit buffer, M-potassium phosphate buffer, pH 6.8 (measured at 50 mM-phosphate), was in chamber 3 of a three-chambered system. Chambers 1 and 2 contained 166.5 ml. of 1 mM-potassium phosphate buffer, pH 6.8, and chamber 3 contained 146 ml. of the limit buffer. The chromatography was conducted in a cold room maintained at 3–5°.

In those experiments in which 240 mg. of protein was applied to the column, the dimensions of the column were increased to 40 cm. $\times$ 1.35 cm. diam. The first two chambers of the Varigrad device contained 200 ml. of starting buffer and the third chamber contained 172 ml. of limit buffer.

Protein determination. Protein concentration in eluate fractions was estimated by solving the equations of Warburg & Christian (1941) for the estimation of protein based on the extinctions at 280 and 260 $m\mu$ (Layne, 1957). The equations were empirically derived by measuring the extinctions of mixtures of yeast enolase and nucleic acid at 280 and 260 $m\mu$. Chromatographic profiles obtained by this method agreed with those obtained by using the Folin-Lowry method of protein determination (Lowry, Rosebrough, Farr & Randall, 1951). Both methods are dependent on the tyrosine and tryptophan content of the proteins.

The major contribution to the extinction at 226.5 $m\mu$ is the peptide bond (Murphy & Kies, 1960). At this wavelength the interference by nucleic acid is minimal. Provided that the extinction of the protein solution at 226.5 $m\mu$ is not greater than 1.0, it was found that the chromatographic profile obtained by determination of the extinction at 226.5 $m\mu$ was almost identical with that obtained by using the equations of Warburg & Christian (1941). In two experiments it was found that the extinction unit at 226.5 $m\mu$ was equivalent to 0.127 ± 0.007 (5.5%) and 0.122 ± 0.005 (4%) mg. of protein/ml. as calculated from the readings at 280 and 260 $m\mu$.

The methods described are not applicable to fractions that contain low-molecular-weight substances that absorb

in the ultraviolet. Therefore substances such as ATP and NAD must be removed.

Nucleic acid determination. This was also determined by the use of empirical equations (Warburg & Christian, 1941). The chromatographic profile obtained by this method was identical with that obtained by determining nucleic acid of the fractions by the method of Logan, Nannell & Rossiter (1953). In the latter method, samples of the tube contents were heated at 90° for 15 min. in 5% (w/v) trichloroacetic acid, the precipitated protein was removed by centrifugation and the nucleic acids were determined by reading the extinction at 268.5 $m\mu$.

Preparation of particle fraction. The procedure was the same as that reported by Brunngraber & Aguilar (1962) for the preparation of brain mitochondria, except that the combined mitochondrial and microsomal fractions were obtained by centrifugation at 105 400g for 30 min. Rotor no. 40 of the Spinco model L ultracentrifuge was used in all experiments.

RESULTS

Chromatography of soluble brain proteins on hydroxyapatite. For the standard experiment, whole rat brains were quickly excised and homogenized in 0.25 M-sucrose. The homogenate was then centrifuged at 105 400g for 30 min. and an amount of supernatant containing 60 mg. of protein (biuret method) applied to the calcium hydroxyapatite column. Unadsorbed proteins were removed by flushing the column with 1 mM-potassium phosphate buffer, pH 6.8. The 'peak' that represents this fraction has been omitted in the chromatograms presented in Figs. 1 and 2. At least 11 protein fractions are reproducibly obtained and these are represented by the numbered peaks (Fig. 1). There are, in addition, two major nucleic acid fractions represented by peaks I and II in the chromatograms (Fig. 1a). The nucleic acids of peak II appear to be an unresolved mixture of two or possibly three components.

Chromatograms obtained over the past 2 years have been highly reproducible. The amounts of protein and nucleic acid eluted from the column by displacement with phosphate are shown in Table 1 and these values have been used to calculate the ratios of protein to nucleic acid presented in Table 2.

Application of 240 mg. of protein to a somewhat larger column yielded chromatograms that were strikingly similar to the one shown in Fig. 1(a). It was found that the dimensions of the calcium hydroxyapatite column are not critical. A ratio of length to width as low as 2 yielded chromatograms that were identical with those achieved with column-dimension ratios as high as 15. This is of practical importance, since the flow rate with shorter and wider columns is considerably faster. Some preparations of calcium hydroxyapatite give extremely low flow rates and the use of shorter

Fig. 1. Chromatograms obtained when soluble extracts from whole rat brain are subjected to column chromatography on calcium hydroxyapatite columns (20 cm. $\times$ 1.3 cm.). The 'run-through' peak is not shown. The proteins and nucleic acids depicted are those that have been adsorbed on the gel and eluted with potassium phosphate buffer, pH 6.8. (a) Chromatogram of supernatant obtained by centrifuging whole rat-brain homogenates prepared in 0.25 M-sucrose for 30 min. at 105 400g. (b) Chromatograms obtained with extracts prepared as in (a), except that the brain was frozen by contact with solid CO₂ and thawed before homogenization. (c) Chromatogram of extracts prepared by homogenizing brain in 20 mM-tris-HCl buffer, pH 8.0, and centrifuging at 105 400g for 30 min.; the residue was re-extracted with water and recentrifuged, and the combined extracts were applied to the column. (d) Chromatography of soluble extracts prepared from the particle fraction (see the text). (e) Same as (a), except that centrifugation was extended to 90 min. (f) Chromatography of supernatant obtained by centrifuging whole rat-brain homogenates prepared in iso-osmotic KCl for 30 min. at 105 400g. —, Concn. of protein; ······, concn. of nucleic acid; -x-x-x-, concn. of phosphate.

Table 1. *Amounts of protein and nucleic acid adsorbed to calcium hydroxyapatite and eluted with potassium phosphate buffer, pH 6.8*

Total protein and nucleic acid eluted from calcium hydroxyapatite columns, the amount of nucleic acid appearing in the fractions corresponding to peaks I and II, and the percentage of the total nucleic acid of peaks I and II that appears in peak I and in peak II, are given for the various chromatographic experiments described in the text.

Experiment	Protein (mg.)	Nucleic acid (mg.)	Nucleic acid in peak I (mg.)	Nucleic acid in peak II (mg.)	Nucleic acid in peak I (%)	Nucleic acid in peak II (%)
8 standard columns (average)	51.80 ± 3.7	1.05 ± 0.07	0.33 ± 0.04	0.72 ± 0.08	32 ± 4	68 ± 4
8 standard columns (range)	44.30–57.90	0.80–1.13	0.29–0.41	0.50–0.82	26–40	60–74
Freezing and thawing whole brain	52.5	0.66	0.28	0.38	42	58
Soluble proteins from particle fraction	58.10	2.49	0.28	2.21	11	89
Tris-HCl extract from whole brain	58.25	1.06	0.34	0.72	32	68
Iso-osmotic KCl extract from whole brain	44.56	0.58	0.35	0.23	60	40
Centrifugation of 0.25 M-sucrose homogenates for 90 min.	47.15	0.43	0.29	0.14	67	33
Homogenate incubated for 2 hr. at 37°	50.21	1.93	0.60	1.33	31	69
Homogenate incubated in O ₂ for 2 hr. at 37°	46.30	1.42	0.35	1.07	25	75

Table 2. *Ratios of protein to nucleic acid*

The data of Table 1 were used to calculate the ratios of total protein to total nucleic acid, total protein to nucleic acid in the fraction corresponding to peak I, and total protein to the amount of nucleic acid in peak II. In addition, the ratio of the amount of protein that is eluted in the fraction corresponding to peak II to the amount of nucleic acid in this fraction is given. The nature of the extract is shown to affect the values of the ratios.

Experiment	Total protein/ total nucleic acid	Total protein/ nucleic acid in peak I	Total protein/ nucleic acid in peak II	Protein in peak II/ nucleic acid in peak II
8 standard columns (average)	49.6 ± 2.9	157.6 ± 15.1	73.4 ± 7.4	21.94 ± 4.0
8 standard columns (range)	42.5–55.4	127.4–196.2	58.7–88.6	15.66–28.4
Freezing and thawing whole brain	79.5	187.5	138.1	45.2
Soluble proteins from particle fraction	23.3	207.5	26.28	13.93
Tris-HCl extract from whole brain	54.9	171.3	80.9	32.80
Iso-osmotic KCl extract from whole brain	76.82	127.3	193.7	57.8
Centrifugation of 0.25 M-sucrose homogenates for 90 min.	109.6	162.5	336.7	77.5
Homogenate incubated for 2 hr. at 37°	26.01	83.68	37.75	14.22
Homogenate incubated in O ₂ for 2 hr. at 37°	32.60	132.22	43.3	14.06

columns can to a large extent compensate for this. The fastest flow rate used in all of the present experiments was 30 ml./hr. To test the effect of flow rate, the rat-brain soluble proteins were applied to a 2 cm. × 1 cm. column of a gel preparation that gave unusually fast flow rates. The two nucleic acid fractions were clearly separated from each other with a flow rate of 100 ml./hr.; however, some of the protein peaks were not as clearly resolved.

Changes in the chromatogram with changes in experimental procedures. Changes in the methods used for the preparation of brain soluble proteins have an effect on the chromatograms obtained when

the extracts are subjected to the same chromatographic procedure as applied to standard preparations. Freezing and thawing the brain after its removal from the animal caused a decline in the amount of nucleic acid eluted from the column. The nucleic acid in peak II showed the greater decrease (Fig. 1b). Since the amount of protein obtained was unchanged, the ratios of protein to nucleic acid (Table 2) increased. Homogenates prepared in iso-osmotic potassium chloride instead of 0.25 M-sucrose yielded chromatograms that indicated that most of the nucleic acid of peak II is missing (Fig. 1f). Centrifugation of sucrose homogenates at 105 400g for 90 min., rather than the

standard 30 min., caused the nucleic acids of peak II to disappear almost completely (Fig. 1e). Supernatants spun for only 30 min. contain ribosomes (Wolfe, 1961). These apparently become bound to the column and release their nucleic acids when the column is washed with dilute phosphate buffer. The presence of particulate matter in such supernatants has been demonstrated by applying them to Sephadex G-25 and exchanging the sucrose in the solvent for 0.15M-potassium chloride by gel filtration. The eluate, containing all of the protein of the extract, became turbid. Clarification of the turbid suspension by centrifugation removes the agglutinated ribosomes as well as the nucleic acids of peak II. The same result is obtained by homogenizing the brain in 0.15M-potassium chloride.

Once the nucleic acids of peak II are eluted from the column, they appear as soluble nucleic acids. Test tubes that contain this material are clear in spite of the presence of high concentrations of phosphate ion.

The procedures that cause the loss of nucleic acid of peak II are those that might be expected to cause sedimentation of ribosomes. These considerations lead to the conclusion that nucleic acid in peak II is ribosomal RNA. Nucleic acids in peak I were not significantly affected by the three procedures described. This material is a constituent of the cell sap, and remains unsedimented even after 90 min. centrifugation at 109 000g. These properties suggest that it may be related to transfer RNA (s-RNA).

It is generally the practice to extract soluble proteins from brain with aqueous solvents at low ionic strength. To obtain chromatograms of such extracts, whole rat brain was homogenized in 20mM-tris-hydrochloric acid buffer, pH 8.0, and centrifuged for 30 min. at 109 000g. The sediment was taken up in water, and rehomogenized and again centrifuged. The combined supernatants were then applied to the calcium hydroxyapatite columns. The chromatogram (Fig. 1c and Tables 1 and 2) appears to be similar to those obtained with the standard columns, except that the heterogeneity of the nucleic acid of peak II is accentuated. This peak is partially resolved into two fractions.

The particle fraction, obtained by differential centrifugation, was extracted with 20mM-tris-hydrochloric acid buffer, pH 8.0, and centrifuged at 109 000g for 30 min. The sediment was re-extracted with water and the centrifugation repeated. The combined supernatants, representing 60 mg. of protein (biuret), were applied to the gel. The ratio of total protein to total nucleic acid (Table 2) eluted from the column is one-half that obtained with the standard supernatant. The extract also contains soluble RNA and this is presumably derived from the nerve endings or axonal fragments

present in this fraction. The ratio of total protein to nucleic acid in peak I is close to the standard value (Table 2). The chromatogram (Fig. 1d) differs from the standard in that the nucleic acid content of peak II is increased threefold. Treatment of the particulate fraction (which is rich in ribosomes since it contains the microsomes) with aqueous solvents solubilized a portion of its nucleic acid. It would be expected that intact ribosomes would sediment in 30 min. in low-density medium. Further, the medium contains electrolytes, which would cause agglutination of ribosomes and cause them to sediment at lower speeds.

The findings with soluble extracts of the particulate fraction are in accord with the conclusion that peaks I and II represent soluble and ribosomal RNA respectively.

Protein material not adsorbed by calcium hydroxyapatite. The amount of protein that is not adsorbed by calcium hydroxyapatite is small. About 6% of the total protein recovered appears in this fraction, together with the small molecules and nucleotides present in the crude soluble brain extracts. Several experiments have indicated that the gel does retard the protein material in relation to the nucleotides, but no separations have been achieved. The proteins in this fraction can be separated from the small molecules by gel filtration. The ratio of the extinctions at 280 and 260 m μ of the protein isolated by this method was close to 1.00, indicating that it contained nucleic acid. The material was exhaustively dialysed and then heated in the presence of 5% trichloroacetic acid at 90° for 15 min., and the precipitated proteins were removed by centrifugation. The supernatant gave positive tests for phosphate, ribose, and absorption at 268.5 m μ . The proteins that are not retained by the calcium hydroxyapatite column also appear to contain phospholipids. The nucleic acid not retained by the gel constitutes the third nucleic acid fraction isolated.

Effect of autolysis on chromatograms. Chromatography of the extract after autolysis revealed that the ribosomal RNA (peak II) was considerably increased (Fig. 2a and Tables 1 and 2), and the protein peak corresponding to peak 10 was absent. The peak corresponding to soluble RNA had lost its usual symmetry. Although the appearance of the chromatogram differed somewhat from that of the standard columns, most of the peaks corresponding to protein fractions 1-9 can be discerned.

The effect of autolysis was tested under oxygen. Fig. 2(b) indicates that the ribosomal RNA of peak II is increased in concentration and that the proteins corresponding to peaks 10 and 11 are no longer discernible. If the ribosomes had remained intact, centrifugation for 90 min. would have caused the disappearance of the nucleic acids corresponding

Fig. 2. Chromatograms obtained with soluble extracts from homogenates that had been subjected to autolysis. (a) Whole rat brain was homogenized in 0.25 M-sucrose (one brain/10 ml.) and incubated in a test tube at 37° for 2 hr.; the homogenate was then centrifuged for 30 min. at 105400g and the supernatant was chromatographed. (b) Whole rat brain was homogenized in a volume of 0.25 M-sucrose equal to the weight of the brains used; the homogenate was then incubated with shaking in the Warburg bath under O₂+CO₂ (95:5) for 2 hr.; the pH of the mixture was 6.9, 7.1, 7.4 and 7.5 at 30, 60, 90 and 120 min. respectively; after the incubation, the homogenate was diluted to the usual concentration by the addition of 0.25 M-sucrose and centrifuged at 105400g for 90 min. (c) Same as (b) except that the incubation was carried out under N₂+CO₂ (95:5). (d) The particulate fraction obtained in (b) was resuspended in 0.25 M-sucrose and subjected to differential centrifugation to obtain the particulate fraction (minus nuclei and cell debris) by procedures similar to those used to obtain the chromatogram shown in Fig. 1(d); the particles were extracted with tris-HCl buffer and water and the combined extracts were subjected to chromatography. —, Concn. of protein; ·····, concn. of nucleic acid.

to peak II (Fig. 1e). Autolysis apparently caused the breakdown of ribosomal RNA so that it was no longer sedimentable by centrifuging for 90 min. The effect appeared to be dependent on aerobic conditions, since autolysis under nitrogen plus carbon dioxide (95:5) was not as effective in raising the amount of ribosomal RNA corresponding to peak II (Fig. 2c).

With the exceptions noted, autolysis appears to have only a small effect on the remainder of the chromatogram. The proteins of the cell sap appear to be fairly stable. This does not appear to be the case with the soluble proteins associated with the

particulate fractions. The particle fraction was obtained after the homogenate had been subjected to the aerobic incubation. Soluble proteins were extracted from the particles with tris-hydrochloric acid buffer and water as before and subjected to chromatography. The chromatogram, depicted in Fig. 2(d), should be compared with that shown in Fig. 1(d). Autolysis caused marked changes and it was no longer possible to identify the usual protein peaks.

Blood proteins. Extracts prepared from whole rat-brain homogenates contain haemoglobin that has become released from erythrocytes. The

amount of this contamination can be minimized if care is taken in killing the animal and excising the brain. Blotting the excised brain with filter paper is a useful procedure. If the extracts contain large amounts of haemoglobin, this protein will appear in those test tubes that contain ribosomal RNA. The ratio of extinctions at 280 and 260m μ for haemoglobin is 1.04; the methods of protein and nucleic acid determination used in this investigation are based on the assumption that this ratio for protein and nucleic acid is 1.75 and 0.49 respectively. The presence of large amounts of haemoglobin can therefore give erroneously high values for ribosomal RNA, as well as for proteins. Small amounts of haemoglobin do not interfere seriously. To demonstrate this point, rat brains were perfused with iso-osmotic sucrose, and the brains excised, homogenized and centrifuged. The extracts obtained were colourless, and, on chromatography, yielded chromatograms identical with those of the standard controls (Fig. 1a).

DISCUSSION

Efforts in this Laboratory have been directed towards the development of preparative procedures permitting a systematic separation of brain macromolecules. The aim is to achieve a simultaneous separation of as many protein or protein complexes as possible from the same starting material. In view of the large number of discrete macromolecules present in tissue, it is obvious that a single procedure will not accomplish this purpose. A sequence of operations is essential; each separation must achieve a separation based on some specific property of the macromolecules. The order of the sequence itself is of importance, since the property of a particular protein is often conditioned by the nature of the mixture of which it is a part. Preferably, the first step should achieve a maximal separation; it should be simple, mild and rigidly reproducible. It should provide as much information as possible so that it might reveal changes in the protein profiles caused by various experimental procedures. This would serve to pinpoint those fractions that have undergone changes and that should receive preference in the application of subsequent steps in the fractionation procedure.

Calcium hydroxyapatite, first described by Tiselius *et al.* (1956), has several advantages as a first step in the proposed sequence. Reproducible chromatograms have been achieved by its use. It allows for 100% recovery of applied materials. If some material is irreversibly bound, the gel can be dissolved in acid and dialysed to recover the adsorbed material. This is an advantage in determining recovery of isotopes in radioactive incor-

poration studies. The amount of such irreversibly bound material is small; in this respect it appears to have an advantage when compared with the cellulose ion-exchangers. Further, high-speed supernatants prepared from tissue homogenates can be applied directly with the knowledge that the small molecules are not adsorbed. No dialysis or gel filtration is required as a preliminary step. This is not true for cellulose ion-exchangers, which will bind substances such as NAD and ATP.

The fact that most of the purified enzyme preparations available today have been carried through a purification step utilizing calcium phosphate as an adsorbent is testimony to its mildness. It was also shown (Brunngraber & Aguilar, 1962) that isoenzymes of lactate dehydrogenase (four peaks) and malate dehydrogenase (two peaks) can be separated by using calcium hydroxyapatite. This gel can also be used to separate nucleic acid components. It is not known whether this separation is due to the separation of the nucleic acids themselves, or the separation of proteins to which these substances are attached. DEAE-cellulose anion-exchangers bind nucleic acid tightly, possibly separating the protein from its bond with the nucleic acid component. Canellakis & Herbert (1960) used calcium hydroxyapatite to separate three ribonucleoproteins from the high-speed supernatants prepared from liver. Chromatography on DEAE-cellulose was utilized to split the nucleic acids from the protein component.

The technique of column chromatography on calcium hydroxyapatite gel was used to study the effects of autolysis on brain tissue homogenates. Earlier, Vrba & Folberger (1958) reported that aerobic incubation of guinea-pig cortex slices in the absence of substrate resulted in a decrease in protein and nucleic acid nitrogen that was accompanied by an increase in ammonia, non-protein nitrogen and lipid nitrogen. In the present investigation aerobic autolysis appears to affect the proteins and nucleic acids that normally appear in the supernatant after centrifuging homogenates prepared in 0.25M-sucrose at 105400g for 90min. in the following ways. First, ribosomal RNA is increased in amount and the increase causes the fraction that corresponds to ribosomal RNA to become symmetrical. Normally, peak II is absent if the homogenate is spun at 90min. or appears as a compound peak if spun at 30min. Secondly, the tail end of the chromatogram shows a precipitous decline of ribosomal RNA (peak II), and this is accompanied by a simultaneous decline of protein concentration in successive eluate fractions. Normally, the ribosomal RNA of peak II is followed by the proteins corresponding to peaks 10 and 11. Procedures described in this investigation may be useful in isolating proteinases or other enzymes that

are active in autolysis, and in isolating the normal substrates for these enzymes.

This investigation was supported, in part, by Public Health Service Grant NB-05023-02 from the National Institutes of Neurological Diseases and Blindness, U.S. Public Health Service. We thank Mr Alva Earley for his technical assistance.

REFERENCES

- Bogoch, S., Rajam, P. C. & Belval, P. C. (1964). *Nature, Lond.*, **204**, 75.
- Bondy, S. C. & Perry, S. V. (1963). *J. Neurochem.* **10**, 593.
- Brunngraber, E. G. & Aguilar, V. (1962). *J. Neurochem.* **9**, 451.
- Canellakis, E. S. & Herbert, E. (1960). *Biochim. biophys. Acta*, **45**, 133.
- Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Logan, J. E., Nannell, W. A. & Rossiter, R. J. (1953). *Biochem. J.* **51**, 470.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 263.
- Mokrasch, L. C. & McGilvery, R. W. (1956). *J. biol. Chem.* **221**, 909.
- Murphy, J. B. & Kies, M. W. (1960). *Biochim. biophys. Acta*, **45**, 382.
- Peterson, E. A. & Sober, H. A. (1959). *Analyt. Chem.* **31**, 857.
- Tiselius, A., Hjerten, S. & Levin, O. (1956). *Arch. Biochem. Biophys.* **65**, 132.
- Vrba, R. & Folberger, J. (1958). *Nature, Lond.*, **182**, 237.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
- Wolfe, L. S. (1961). *Biochem. J.* **79**, 348.