Isolation and Properties of Polyribosomes from Cerebral Cortex*

Anthony T. Campagnoni and H. R. Mahler†

ABSTRACT: A method is described for the preparation of ribonucleoprotein particles from rat cortical tissue without the use of detergent. The resulting population is polyribosomal in character with $s^0_{20,w}$ values of 76, 118, 148, 176, 193, and 238 for the first six members. Sucrose density gradient analysis and electron microscopy support the assignment of the *s* values. *In vitro* protein synthesis was studied using these ribonucleoprotein particles. Optimal Mg^{2+} concentration was about 10 mM. Ca^{2+} had little effect when added to the standard incorporating system; however, upon replacing Mg^{2+} with Ca^{2+}, with the total divalent metal ion concentration at 10 mM, an increase in [^{14}C]leucine incorporation was observed over a fairly broad range followed by a rapid decline in incorporation upon further replacement of Mg^{2+} by Ca^{2+}. The effects of K^+, NH_4^+, and Na^+ were studied and variations in activity between NH_4^+ and either K^+ or Na^+ appeared merely to be a linear function of the mole fraction of monovalent ions. Variations in the K^+:Na^+ ratio, with the total monovalent cation concentration at 140 mM, produced a different effect. Two maxima were observed; one at 124–128 mM K^+:16–12 mM Na^+ and another smaller one at about 90 mM K^+:50 mM Na^+. Finally, the incorporating system appeared to be stimulated by a mixture of 19 amino acids. About one-half of this stimulation could be accounted for by a mixture of the seven amino acids which have been shown to constitute over 80% of the free amino compounds found in (guinea pig) brains. Thus three regulatory mechanisms appear to be operative in this system: Ca^{2+}:Mg^{2+}, Na^+:K^+, and amino acids.

The concept that poly(ribo)somes, aggregates of ribonucleoprotein particles held together by strands of RNA, are the functional units in protein synthesis in most cells has been supported by an imposing body of experimental evidence (Warner *et al.*, 1963; Gierer, 1963; Wettstein *et al.*, 1963; Penman *et al.*, 1963).

In animal cells ribosomes and polysomes exist in two forms: relatively free in the cytoplasm or attached to the endoplasmic reticulum. Commonly, ribosomal preparations have entailed the use of detergents, such as sodium deoxycholate, for the liberation of the bound particles (Wettstein *et al.*, 1963; Penman *et al.*, 1963; Korner and Munro, 1963; Palade and Siekevitz, 1963; Korner, 1961; Campbell *et al.*, 1966). The possibility exists, however, that this treatment may modify or disrupt the aggregates existing *in vivo*, and in order to forestall this, several investigators have developed alternative methods for the isolation of polysomes that do not use detergents (Webb *et al.*, 1964; Marbaix and Burny, 1964; Arnstein and Cox, 1963; Bont *et al.*, 1965).

In rat brain cortex, furthermore, as suggested by electron micrographs of thin sections, neuronal cells may possess a higher proportion of free cytoplasmic than of reticulum-bound ribosomes, whereas the reverse appears to hold true for the glial cells that surround these neurons (Ekholm and Hyden, 1965; Gray, 1964; C. Cotman, unpublished observations). Thus, preparations of polysomes obtained from rat brain cortices without the use of detergents would presumably reflect the situation *in vivo* and be primarily neuronal in origin.

We now wish to report on the isolation of such polysomes and on their physical properties. We have also studied their capabilities in protein synthesis *in vitro*, especially with regard to their responses to environmental factors such as cations and amino acids.

Methods

Preparation of Polyribosomes. Polyribosomes were prepared by a modification of the method of Bont *et al.* (1965). White, male, Sprague–Dawley rats (Simonsen Labs, White Bear Lake, Minn.), 18–24 days old, were stunned and decapitated. The cerebral cortices were removed and homogenized in 2.5 ml of medium P (0.05 M Tris–0.025 M KCl–0.01 M magnesium acetate–0.35 M sucrose, pH 7.6)/g of cortex by 10 up-and-down passes in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged in the no. 30 rotor of the Spinco Model L ultracentrifuge at 15,000 rpm for 15 min (R_{av} = 19,600*g*), and the supernatant was removed, layered over 2.0 M sucrose (in medium P minus sucrose), and centrifuged for 3.5 hr at 47,500 rpm in the no. 50 rotor of the Spinco Model L (R_{av} = 136,000*g*). The supernatant

* From the Chemical Laboratory, Indiana University, Bloomington, Indiana. Contribution No. 1411. *Received November 4, 1966.*

† Research Career Award PHS GM-05060 of the U. S. Public Health Service. This research was supported by Research Grant GM-4855 from the National Science Foundation and Contract No. ONR 908-20 by the Office of Naval Research.

TABLE I: Size Characterization of Polyribosomes from Rat Brain Coretx.

Peak	Electron Micrograph	$s^0_{20,w}$ (S) Analytical Ultracentrifuge	$s^0_{20,w}$ (S) Sucrose Density Gradient	$s^0_{20,w}$ (S) Least-Mean-Square Slope[a]
First	Monomers	76 ± 2	76[b]	−1.26
Second	Dimers	118 ± 3	111 ± 3	−2.57
Third	Trimers	148 ± 6	141 ± 4	−3.53
Fourth	Tetramers	176 ± 7	172 ± 7	−4.60
Fifth	Pentamers	194 ± 8	196 ± 9	−4.97
Sixth	—	261 ± 9 (238 ± 3)[c]	220 ± 11	−9.39 (−7.64)[c]

[a] Δs/mg of ribosomal protein. [b] Since no standards were run, this peak was chosen to have an s value of 76 and the other s values were calculated using this as the standard. [c] Values in parentheses indicate those calculated by disregarding one point on the $s_{20,w}$ *vs.* c curve for the sixth peak. This point was inordinately high; consequently the value of 261 for this peak is probably higher than it should be. The value in parentheses probably more closely approximates the true $s^0_{20,w}$.

was carefully syringed off down to 1 cm from the 2.0 M sucrose interface and used for the isolation of pH 5 enzymes. The clear, translucent polysome pellets were rinsed twice with medium P minus sucrose, resuspended in 1 ml of medium P/g of cortex used originally, and centrifuged for 90 min at 136,000*g*. The pellets from this centrifugation were then resuspended in medium C (0.05 M Tris–0.025 M KCl–0.001 M magnesium acetate, pH 7.6) for use on sucrose density gradients, analysis in the analytical ultracentrifuge, or incubations.

Preparation of pH 5 Enzymes. pH 5 enzymes were prepared by adjusting the pH of the 136,000*g* supernatant to pH 5.2 with 1.0 M acetic acid. The precipitate was then collected by centrifugation at 15,000*g* for 30 min and resuspended in medium C for use in incubations.

Sucrose Density Gradients. Sucrose gradients (15–40%) were prepared in an apparatus like that described by Salo and Kouns (1965). Sucrose solutions were prepared in medium C. About 2 mg of polyribosomes was usually applied to 24-ml gradients layered over 5.0 ml of 2.0 M sucrose (in medium C). Gradients were centrifuged at 22,000 rpm for 2 hr in the SW-25 rotor of the Spinco Model L-2 ultracentrifuge at 4°. The optical density of the gradients at 260 mμ was monitored continuously with a flow-cell attachment to a Gilford recording spectrophotometer, Model 2000.

Analytical Ultracentrifugal Analysis. Analytical ultracentrifugal analysis using schlieren optics was performed on polysomal preparations at concentrations ranging from 2 to 18 mg of ribosomal protein/ml in a Spinco Model E ultracentrifuge equipped with a temperature-control unit. All runs were performed at 4° and 19,160 rpm and the polysomal suspension was in medium C. s values were corrected to 20° in water: the viscosity and density of the solvent were assumed to be those of water at the temperature of the run and were $\eta = 1.5674$ cp and $d = 1.000$ g/cc. A partial specific volume of 0.66 ml/g was assumed for all polysomal aggregates (Petermann, 1964). Plots of $s^0_{20,w}$ *vs.* concentration were then constructed, and least-squares straight lines were extrapolated to zero concentration to yield values for each peak.

Electron Microscopy. Polyribosomal fractions from the sucrose gradients were diluted with 22% sucrose buffered with 0.01 M KCl–0.010 M Tris–0.0015 M $MgCl_2$ at pH 7.4 (Slater *et al.*, 1963). For shadowing, samples were deposited on carbon-coated grids and washed with decreasing concentrations of sucrose solutions, ending with buffer washes. The samples were then platinum shadowed and photographed.

Cell-Free-Incorporating Systems. The normal incorporation system consisted of the following components: polysomes, ~0.1 mg; pH 5 enzymes, ~0.2 mg; magnesium acetate, 10 mM; NaCl, 40 mM; KCl, 100 mM; glutathione (Sigma), 6 mM; ATP[1] (Sigma), 5 mM; GTP (Sigma), 1 mM; Tris, 20 mM; sucrose, 0.25 M; and [^{14}C]leucine (Schwarz, 200–240 mc/mmole), 0.5 μc. Four types of amino acid mixtures were made up and designated as follows: 19 aa, 19 aa + GABA, 7 aa (Val), and 7 aa (w). Final concentrations of all added amino acids were 0.2 mM with respect to each: (1) 19 aa: valine, methionine, tyrosine, cysteine, cystine, glycine, threonine, alanine, hydroxyproline, serine, asparagine, glutamine, histidine, arginine, lysine, proline, aspartic acid, glutamic acid, and tryptophan; (2) 19 aa + GABA: the above plus γ-aminobutyric acid; (3) 7 aa (Val): valine, tyrosine, histidine, tryptophan, methionine, proline, and cysteine; and (4) 7 aa (w): glutamic acid, aspartic acid, γ-aminobutyric acid, glycine, serine, alanine, and threonine.

When an ATP-generating system was being tested, 10 μg of pyruvate kinase (Boehringer) and 1.6 mM

[1] Abbreviations used: ATP and GTP, adenosine and guanosine triphosphates; GABA, γ-aminobutyric acid; aa, amino acid; TCA, trichloroacetic acid; RNP, ribonucleoprotein.

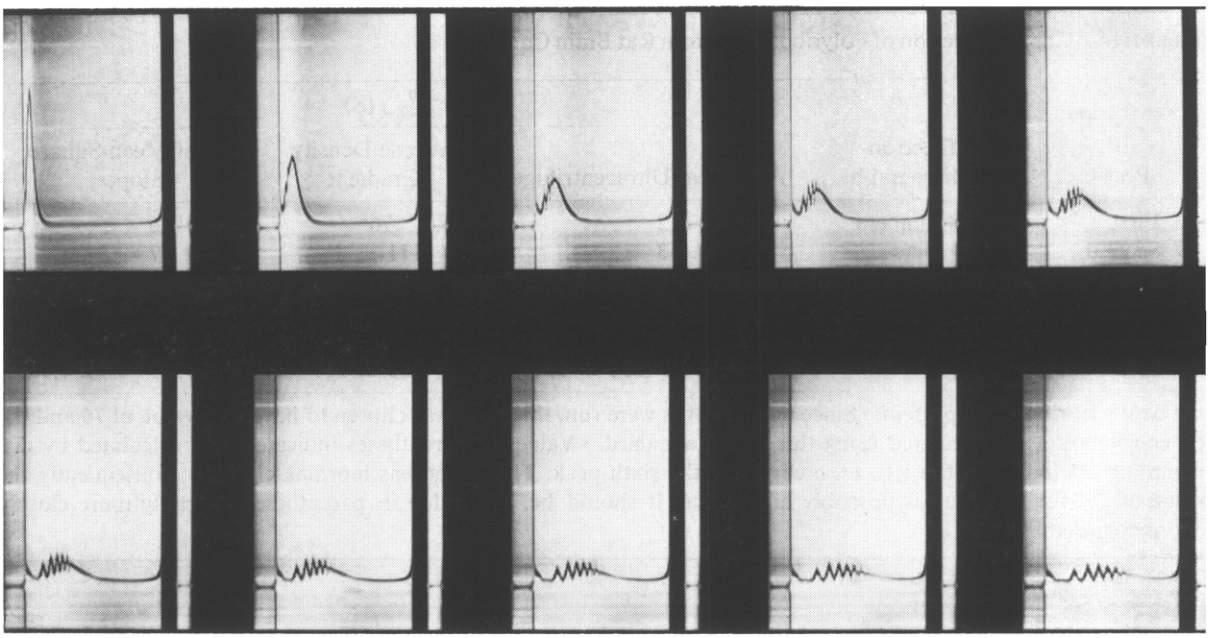

FIGURE 1: Schlieren pattern of polysomal preparation: 4.9 mg/ml, 4°, 19,160 rpm in medium C, time interval 4 min, Spinco Model E.

phosphoenolpyruvate (Sigma) were used. Total volume of incubation mixtures was 1.0 ml.

Incubations were carried out under the following conditions. All ions and other components except pH 5 enzymes and polysomes were mixed at 0°, and then pH 5 enzymes were added followed by the immediate addition of polyribosomes. For zero times the reaction was immediately terminated by making the incubation solution 0.1 M in NaOH. Normal incubations were carried out for 60 min at 37°.

At the end of this time each tube was made 0.1 M in NaOH and mixed thoroughly. Then 0.1 ml of the

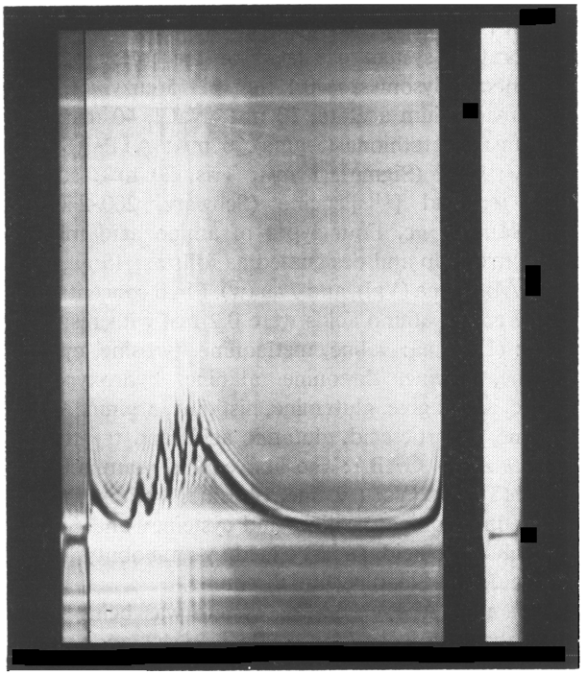

FIGURE 2: Schlieren pattern of polyribosomal preparation 24 min after start of run: 11 mg/ml, 19,160 rpm, 4°, Spinco Model E.

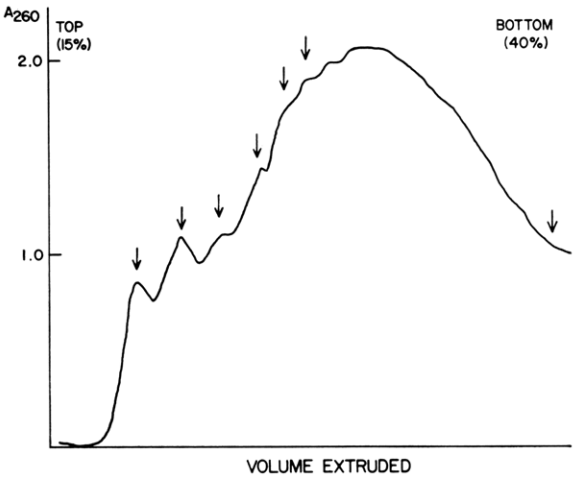

FIGURE 3: Sucrose density gradient profile of polyribosomes. Sucrose gradients were centrifuged for 2 hr at 22,000 rpm in the SW-25 rotor of the Spinco Model L-2 ultracentrifuge at 4°. Approximately 2 mg/ml of ribosomal protein was applied to each gradient. Arrows indicate the points at which $s^0_{20,w}$ values were calculated and/or removed for electron microscopic observation.

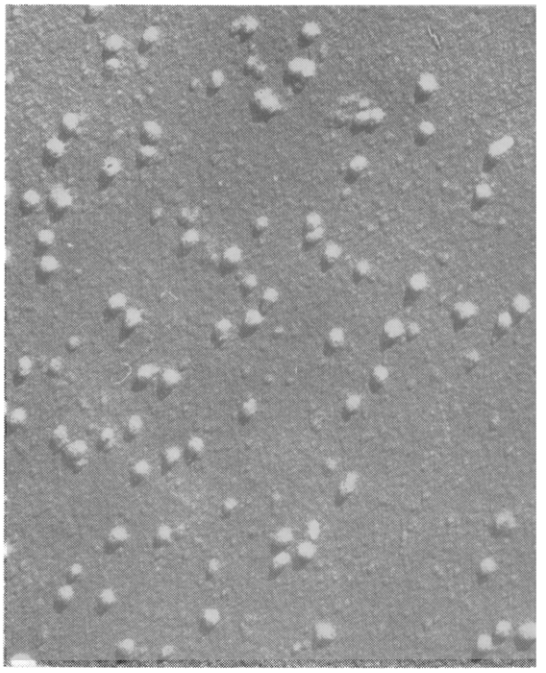

PLATE 1: Electron micrograph of the first peak. This shows a large population of monomers, platinum shadowed, magnification 63,000×.

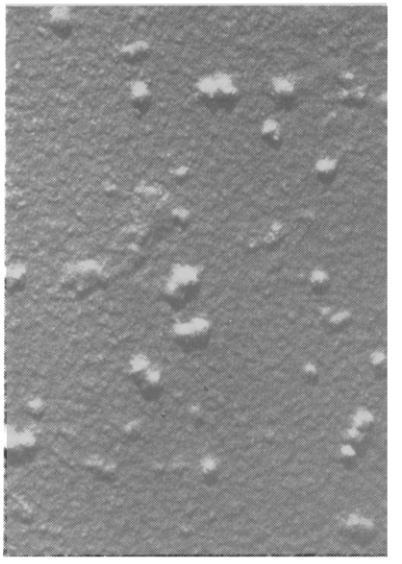

PLATE 2: Electron micrograph of the second peak. A large proportion of dimers is seen in this peak, platinum shadowed, magnification 63,000×.

mixture was plated onto filter paper disks and prepared for counting according to the method of Mans and Novelli (1961). A Packard Tri-Carb liquid scintillation counter, Model 3324, was used. All incorporations were corrected for zero times. Protein was analyzed by the method of Lowry *et al.* (1951). Results are expressed as micromicromoles of leucine incorporated per milligram of ribosomal protein.

Results

Size Characterization of Polyribosomes. The results of the sedimentation analyses are shown in Table I. The $s^0_{20,w}$ values are calculated from several determinations at concentrations ranging from 2 to 18 mg of ribosomal protein/ml by extrapolation to zero concentration as described under Methods. All regression lines were fitted by the method of least squares.

Plots of $s_{20,w}$ *vs.* concentration instead of $1/s_{20,w}$ *vs.* concentration were chosen for extrapolation to zero concentration because of the strong and variable dependence of s for the larger polysomal aggregates upon concentration (see least-mean-square slopes in Table I; Schachman, 1959).

The $s^0_{20,w}$ value for hexasomes appears likely to be too high due to one extremely high point which displaced the curve. The values in parentheses were obtained by disregarding this point. The sixth peak was difficult to identify at concentrations below 8 mg/ml. Therefore, only five experimental points were determined. For peaks 1–4, nine points were determined and for peak 5, seven points were determined. In Table I we also compare sedimentation coefficients determined from sucrose density gradients with those from the analytical ultracentrifugal runs. Good agreement is observed.

Figure 1 shows a series of schlieren patterns of polyribosomes at a concentration of 4.9 mg/ml.

Figure 2 represents another Schlieren pattern (at 11 mg/ml) photographed just before the splitting of the sixth peak while Figure 3 represents a typical sucrose density gradient profile. There is a striking similarity between these two figures, both indicating

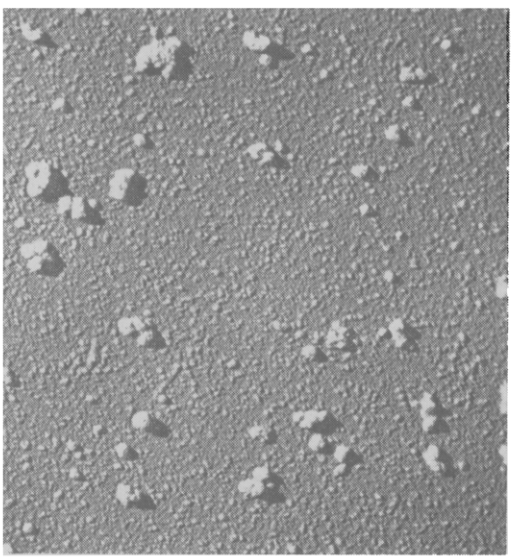

PLATE 3: Electron micrograph of third peak. A considerable number of trimers is seen, magnification 63,000×.

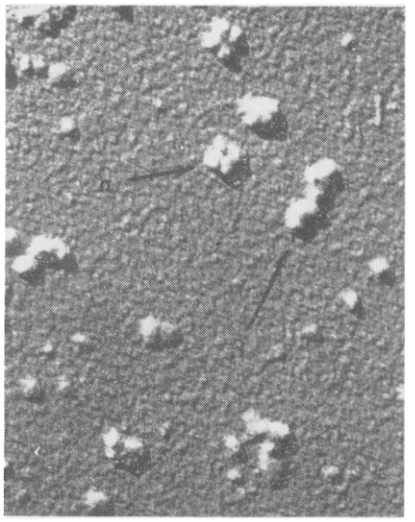

PLATE 4: Electron micrograph of fourth peak. The arrows indicate tetramers. Two types of tetramers are seen: α (clusters) and β (linear arrays); platinum shadowed, magnification 63,000×.

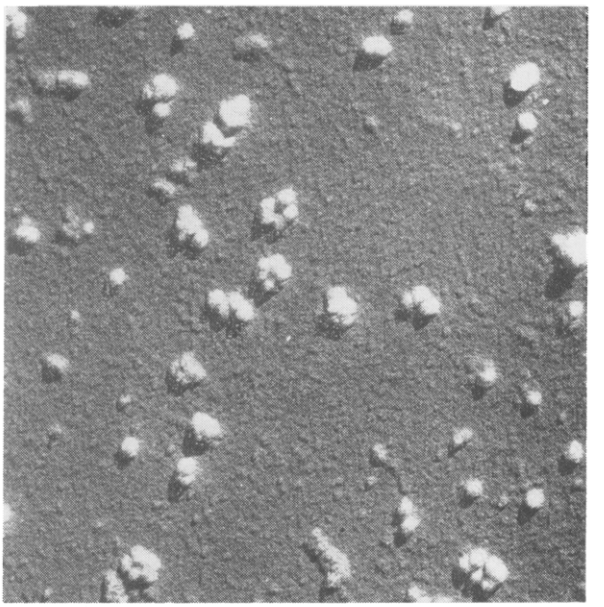

PLATE 5: Electron micrograph of fifth peak. The most common large form is the pentagonal cluster, as shown, magnification 63,000×.

a rather large proportion in the population of polyribosomes composed of more than three units.

At the points indicated in Figure 3 samples were removed and examined in the electron microscope. Plates 1–6 show electron micrographs of these various peaks. *s* values were calculated from the gradient analysis assuming that the first peak had a sedimentation value of 76.[2]

The electron micrographs further support the assignment of polysomal size. No problems were ever encountered identifying the first peak as monomers; however, larger aggregates were more difficult to correlate due to degradation of these structures to smaller units and the obvious overlap of the higher peaks. Polysomal structures are extremely sensitive to osmotic shock and they must be washed carefully with sucrose solutions of decreasing concentration each differing from the preceding one by no more than 5% (H. S. Slayter and A. Rich, personal communication). The electron micrograph (Plate 6) of the tail end of the gradient shows the heterogeneous, large aggregates which sediment in this region.

Requirements for Cell-Free Incorporation. Figure 4 shows the time course for the polyribosomal incorporation system. Maximum incorporation is reached after about 30 min and remains steady for at least 80 min subsequent to that.

In Table II are listed the requirements of the system for various factors. The system does not exhibit an absolute requirement for GTP or ATP; however, maximal activity is grossly inhibited on omission of ATP and substantially inhibited when GTP is omitted. The system has an absolute requirement for polyribosomes and pH 5 enzymes and no requirement for an ATP-generating system.

Several experiments were performed to study the effect of varying concentrations of pH 5 enzymes with constant polysomal concentrations and vice

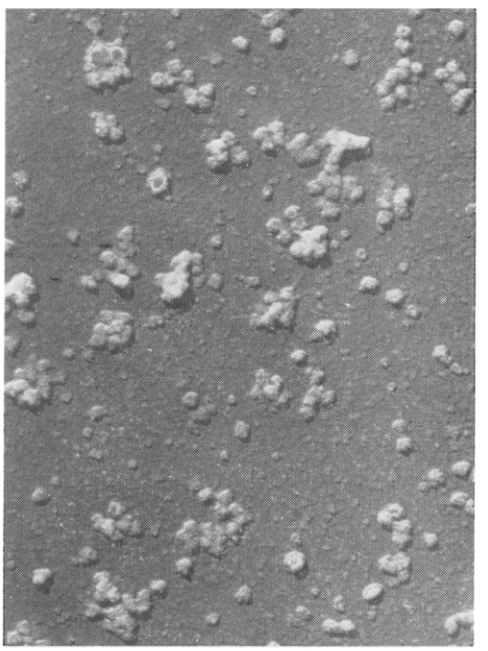

PLATE 6: Electron micrograph of tail end of gradient. Note large aggregates of polysomes, platinum shadowed, magnification 63,000×.

[2] Brief exposure to ribonuclease resulted in elimination of all peaks save this first one.

TABLE II: Requirements of Polyribosomal-Incorporating System.

	$\mu\mu$moles of [^{14}C]-Leucine Inc.	% of Control
Control	123	100
Control − ATP	44	36
Control − GTP	79	64
Control − pH 5 enzymes	10	8
Control + ATP-generating system	132	107
Control − polyribosomes	0	0

versa. The results are shown in Figure 5. It appears that at concentrations of pH 5 enzymes around 200 μg/ml incorporation of [^{14}C]leucine is an insensitive function of polysomal concentration above 50 μg/ml.

Effects of Inhibitors. The effects of puromycin, cycloheximide, chloramphenicol, irehdiamine, and anhydrochlortetracycline were tested in the polysomal incorporation system (Figures 6 and 7). No significant effect was observed when chloramphenicol or anhydrochlortetracycline was added to the incubation medium. Irehdiamine at low concentrations appeared to stimulate incorporation very slightly, but at twice that concentration it inhibited slightly. Cycloheximide was inhibitory at the highest concentrations tested and puromycin definitely inhibited incorporation by about 75% at 80 μg/ml (Figure 7) and 50% at 10 μg/ml.

Ion Requirements. The activity of the polyribosomal system was investigated with respect to effects by various monovalent and divalent cations. The system has an absolute requirement for a divalent cation. The Mg^{2+} concentration curve is plotted in Figure 8. The curve exhibits a fairly broad maximum between 6 and 10 mM Mg^{2+} concentration.

The effect of Ca^{2+} on the system was studied in two ways: first, by simply adding Ca^{2+} in different concentrations to a normal incubation medium containing 10 mM Mg^{2+}. The second method was to keep the total divalent cation concentration constant at 10 mM and vary the proportions of Ca^{2+}:Mg^{2+} (Figure 9). As Ca^{2+} is added to the normal medium slight inhibition occurs up to 10 mM Ca^{2+}. It can be seen from these figures that severe inhibition occurs as Mg^{2+} is replaced by Ca^{2+} as soon as Mg^{2+} is reduced below 5 mM; however, Ca^{2+} at low concentrations appears to stimulate protein synthesis when substituted for Mg^{2+} (up to about one-half Mg^{2+} concentration). Thus, it does not appear that Ca^{2+} can substitute for Mg^{2+}.

The effects of various concentrations of monovalent cations were studied and are plotted in Figures 10–12. Concentration of total monovalent cations was kept at 140 mM and the proportion of cations varied.

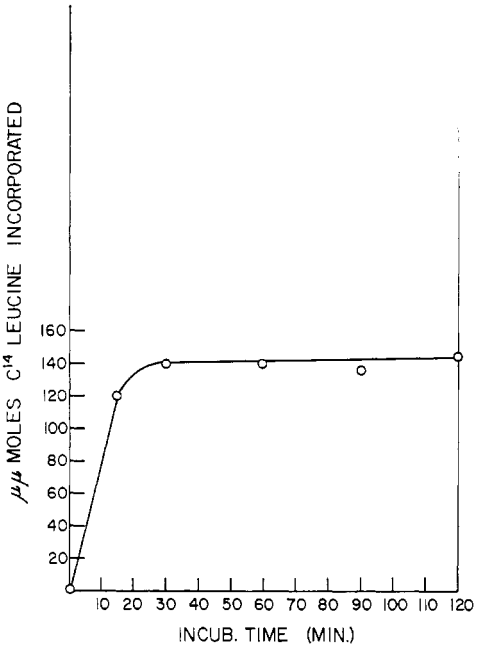

FIGURE 4: Time course of standard polyribosomal incorporation system (Methods).

NH_4^+ (140 mM) appears to lead to highest activity while 140 mM Na^+ yields lowest activity. Mixtures of NH_4^+:K^+ and NH_4^+:Na^+ generate linear functional dependence between the levels of activity of 140 mM NH_4^+, 140 mM K^+, and 140 mM Na^+, respectively. However, this situation does not prevail when Na^+ and K^+ concentrations are varied. Figure 11 represents a composite of six separate experiments. In all cases except one there appeared to be a maximum in the range 120–140 mM K^+ followed by a minimum followed by another smaller maximum which ranges from 90 to 60 mM K^+. This is reflected in the composite graph shown in Figure 11.

The apparent peak activity between 120 and 140 mM K^+ was investigated further by varying the K^+:Na^+ in smaller increments between 120 and 140 mM K^+ (Figure 12). The composite pictures of four experiments is presented in Figure 12. From these plots it can be seen that a maximum indeed occurs between 124 and 128 mM K^+.

Effect of Amino Acids on the Incorporating System. The effect of several combinations of amino acids is represented in Table III. Mixtures of 19 amino acids and 19 amino acids + GABA never failed to stimulate incorporation; however, the actual amount of stimulation varied considerably from preparation to preparation. The mixture of amino acids containing GABA was a bit more erratic in its behavior. In the former case stimulation ranged from 103 to 297% in ten experiments and in the latter case the range was 114–344% in five experiments. Substitution of phenylalanine plus isoleucine for hydroxyproline in the mixture of 19 aa had no effect.

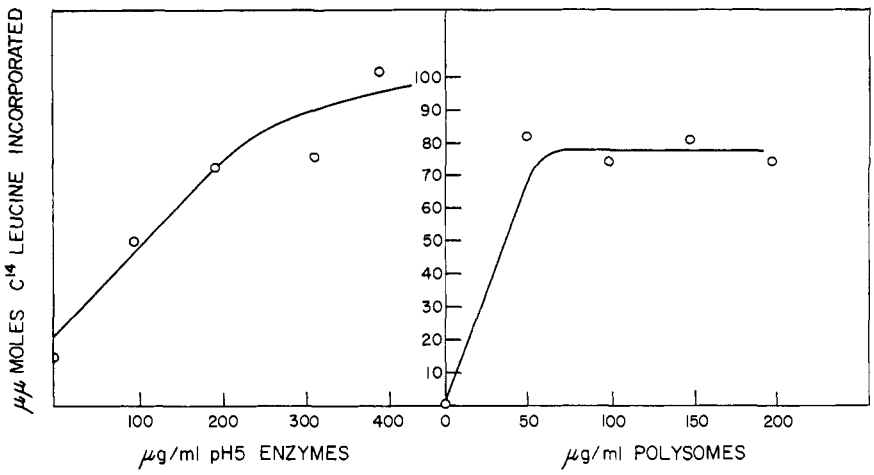

FIGURE 5: Relationship of varying concentrations of polyribosomes and pH 5 enzymes. (left) Concentration of ribosomes held constant at 100 μg/ml. (right) Concentration of pH 5 enzymes held constant at 200 μg/ml.

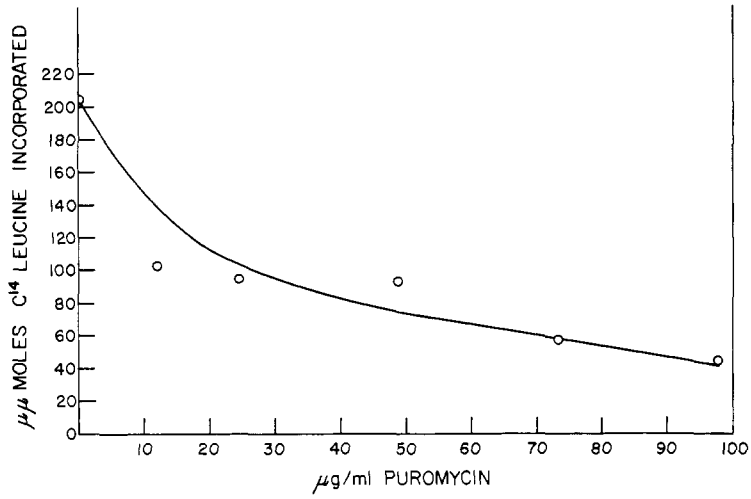

FIGURE 6: Effect of puromycin on the standard polyribosomal-incorporating system.

Less variability in stimulation or inhibition occurred when fewer amino acids were used. Mangan and Whittaker (1966) have reported that seven amino acids account for over 80% of the free amino acid content in guinea pig brain. These seven amino acids are designated 7 aa (w). In four out of five experiments they were found to account for about 50% of the stimulation due to 19 aa. In one experiment they inhibited by about 17%. The averages of all five experiments are shown in Table III.

Seven amino acids were chosen at random to decide whether the stimulation by 7 aa (w) was specific to these amino acids. It was observed that these, abbreviated 7 aa (Val), were consistently inhibitory by about 25%. Several combinations of the amino acids comprising the 7 aa (w) and 7 aa (Val) mixtures were tested, and it was observed that in general the stimulation or inhibition by the mixtures was simply an additive function of the amino acids comprising them. That is, the total stimulation by 7 aa (w) was equal to the individual stimulation of Asp, Glu, GABA + Gly, Ala + Ser, and Thr. In general, Glu, Asp, GABA, Gly, Ala, Ser, Thr, Trp, and Tyr were stimulatory and Val, Cys, Met, Pro, His, and Ile were inhibitory. Nine amino acids known to be essential to the rat's diet were tested and these were consistently inhibitory to incorporation by about 50%.

Discussion

Stimulation of [^{14}C]leucine incorporation by other amino acids has been reported in brain systems by several investigators (Clouet *et al.*, 1966; Stenzel *et al.*, 1966; Murthy and Rappoport, 1965). A mixture of 19 or 20 amino acids has proven to be stimulatory in our system also. The effect of amino acids appears

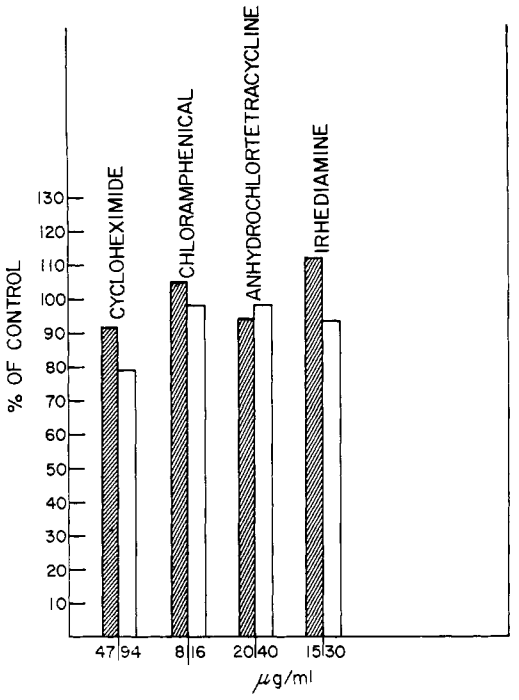

FIGURE 7: Effect of various inhibitors upon the standard incorporating system.

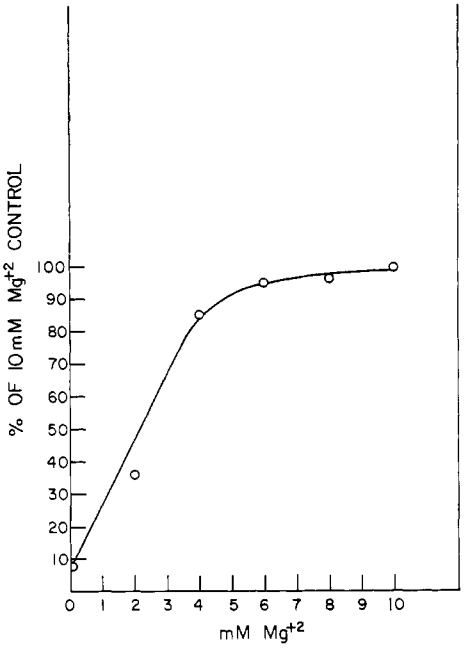

FIGURE 8: Effect of magnesium acetate on the polyribosomal-incorporating system. Mg^{2+} concentration was varied from 0 to 10 mM.

to be simply a function of the stimulatory ability of the individual amino acids and cannot be ascribed to a single amino acid as Stenzel *et al.* (1966) have reported with respect to glutamate in their system. It is of interest to note that the seven amino acids which have been reported to constitute 80% of the free amino compounds present in guinea pig brain (Mangan and Whittaker, 1966) provide close to 50% of the stimulation observed by a mixture of 19 amino acids. It also is of interest to note that the nine essential amino acids inhibit incorporation by 50%. The fact that stimulation by 19 amino acids varies so considerably is not too surprising since the pH 5 fraction is a rather crude mixture of several components, not the least of which is constituted by amino acids bound and free, and this would contribute heavily to this variability.

Little work has been reported until now on sedimentation coefficients of rat brain polysomal aggregates and none on their concentration dependence. The least-mean-square slopes in Table I give this concentration dependence of ribosomes in $\Delta s_{20,w}$/mg of ribosomal protein. There is a marked increase in this slope as the size of the aggregates increases.

While this paper was in preparation Zomzely *et al.* (1966) reported $s_{20,w}$ values of polysomal aggregates isolated from rat brain cerebral cortex by the detergent method. When our $s_{20,w}$ values are corrected to the concentration used in their studies on the first three peaks (3 mg/ml), the two sets of values are in very close agreement (theirs: 73.1 ± 0.9, 108.6 ± 1.0, and 138.3 ± 0.7; ours: 72 ± 2, 110 ± 3, and 137 ± 6). The $s_{20,w}$ values of polysomes isolated from rat cortical tissue reported here appear to be slightly lower than those calculated for similar particles from rat liver (Pfuderer *et al.*, 1965).

Our preparations are clearly polysomal in character as is evidenced by their sucrose density-gradient profiles. Zomzely *et al.* (1966) have reported that at low Mg^{2+} and K^+ concentrations polyribosomal preparations become increasingly dissociated to smaller units (monomers and dimers). Our preparations, isolated at relatively low concentrations of these ions, are principally composed of polyribosomes containing three units and more. It is conceivable, then, that if particles had been isolated and studied at higher concentrations of Mg^{2+} and K^+ their content of monosomes and

TABLE III: Effect of Amino Acids on Standard Incorporating System.

Amino Acid Combination	Av % of Control	No. of Expt	Range
19 aa	200	10	103–297
19 aa + GABA	197	5	114–344
7 aa (w)	145	5	83–184
7 aa (val)	76	4	68–90
9 essential amino acids[a]	48	3	47–50

[a] Arg, His, Lys, Trp, Phe, Cys, Thr, Ile, and Val.

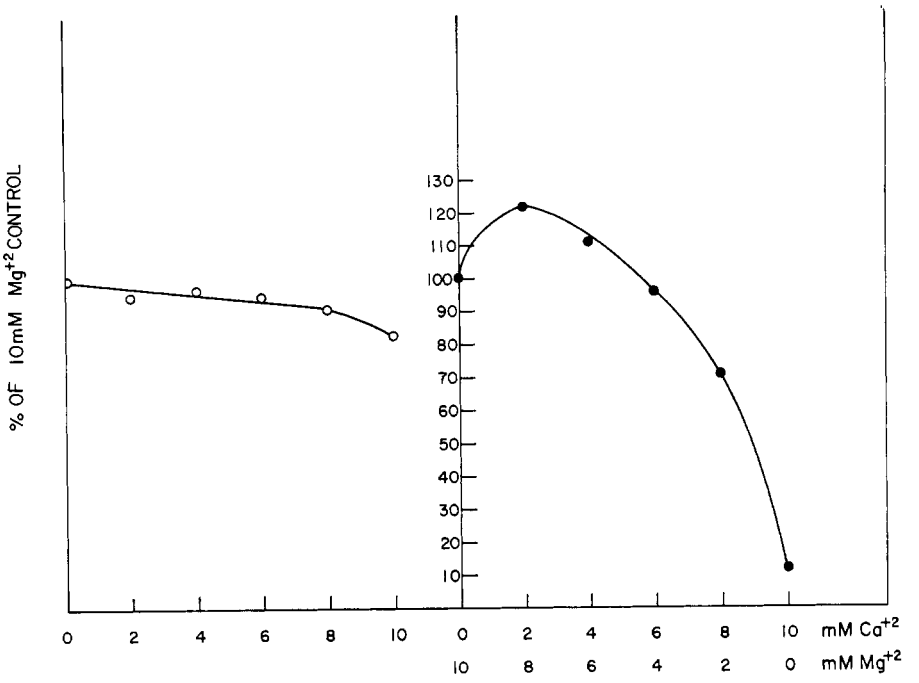

FIGURE 9: Effect of $Ca(NO_3)_2$ on standard incorporating system. (left) Ca^{2+} was added to the standard incorporating system (which contains 10 mM Mg^{2+}) up to a total concentration of 10 mM. (right) Ca^{2+} replaces Mg^{2+} while maintaining the total monovalent cation concentration at 10 mM.

disomes could be further decreased. This is unlikely, however, since aggregation and disaggregation is in all probability solely a function of the number of monomers present and deoxycholate-treated preparations would be more likely to contain a higher number of monomers and hence would be more subject to aggregation–disaggregation effects than a nondeoxycholate-treated preparation such as ours. Our gradients in 1 mM Mg^{2+} and 25 mM K^+ appear to contain a proportion of larger polysomes, at least as high as, or higher than, those reported by Zomzely *et al.* (1966) at 10 mM Mg^{2+} and 100 mM K^+. Thus the more stringent ion effect reported by them may be referable to the more heterogeneous population or some latent damage introduced by the use of detergents.

Density gradient profiles always were of the same general shape as shown in Figure 3 although the heights of the individual peaks varied slightly from preparation to preparation. In only one case was a strong peak evident at the beginning of the gradient (at the sucrose–supernatant interface) and this, when observed under the electron microscope, appeared not to be ribosomal in character but was caused by extraneous protein. The lack of such a peak in all other gradients is probably an indication of the purity of the polyribosomes with respect to adhering protein. The symmetry of the peaks in the schlieren patterns and the absolute requirement of the incorporation system for pH 5 enzymes are added support for this last statement.

Electron micrographs of the various peaks in the gradient indicate the size of the clusters of polysomes comprising these peaks. Micrographs of the higher peaks were not easily obtained until it was realized that the polysomes were sensitive to osmotic shock unless the special precautions described were taken. Pentasomes appeared to be more stable to osmotic shock than other structures. Fairly good micrographs of these pentagonal clusters were obtained even prior to resorting to the special washing techniques. Pentagonal aggregates also appear to be the most prominent structures in Deiter's neurons *in vivo* (Ekholm and Hyden, 1965). Electron micrographs of polyribosomes sedimenting at the bottom of the gradient showed extremely large aggregates upward to about 20 units. Similar forms also appear *in vivo* (Ekholm and Hyden, 1965).

The requirements of this particular polysomal system do not appear to depart widely in many respects from those reported for several other brain systems (Herriman and Hunter, 1965; Stenzel *et al.*, 1966; Murthy and Rappoport, 1965; Clouet *et al.*, 1966; Zomzely *et al.*, 1964). The time course for leucine incorporation into TCA-precipitable material is very similar to that reported by Herriman and Hunter (1965) and Stenzel *et al.* (1966), with maximal activity occurring after about 30 min.

It was found that Mg^{2+} was maximally stimulatory at 10 mM but very high activity (>90%) extended over a wide range from 6 to 10 mM. This is in accordance with results obtained by several other investigators (Murthy and Rappoport, 1965; Clouet *et al.*, 1966; Zomzely *et al.*, 1964). We have not studied the whole

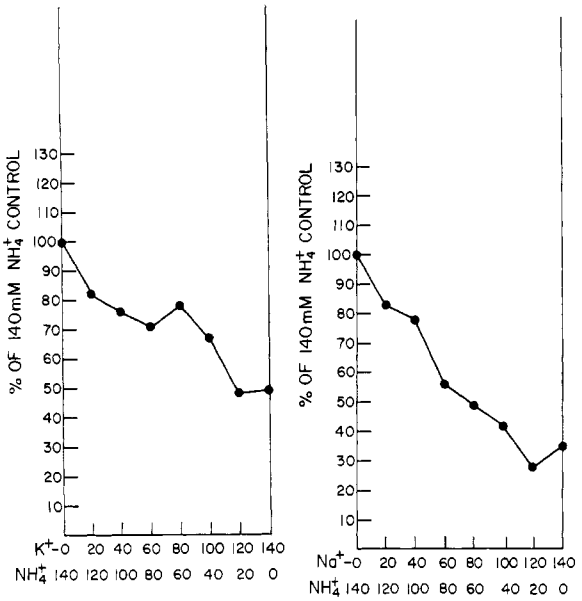

FIGURE 10: Effect of NaCl and KCl on ammonium acetate. Total monovalent cation concentration was held constant at 140 mM. Data are represented as the per cent activity at each concentration with respect to the activity of the 140 mM NH_4^+ control.

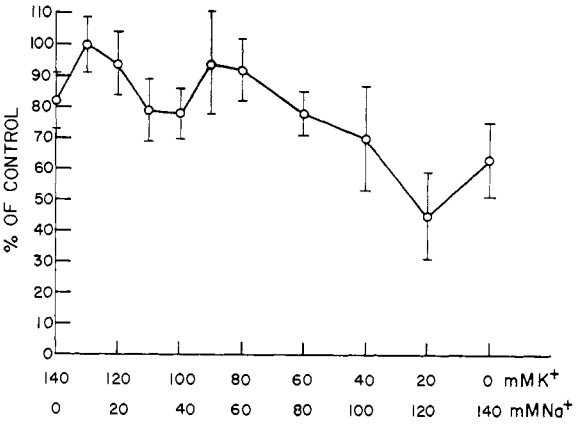

FIGURE 11: Composite of six experiments showing the effect of varying the concentrations of KCl and NaCl while maintaining the total monovalent cation concentration at 14 mM. Points are expressed as per cent of 130 mM K^+ control ± standard error of the mean.

curve beyond 10 mM; however, occasionally 20 mM Mg^{2+} was tested and incorporation was found to be considerably reduced compared to control values (Clouet *et al.*, 1966; Zomzely *et al.*, 1964). The system does not appear to be as sensitive to omission of GTP or ATP as are other brain systems (Stenzel *et al.*, 1966; Murthy and Rappoport, 1965; Zomzely *et al.*, 1964), nor does it have a requirement for an ATP-generating system (Herriman and Hunter, 1965; Clouet *et al.*, 1966; Murthy and Rappoport, 1965; Stenzel *et al.*, 1966; Zomzely *et al.*, 1964).

Puromycin, a known inhibitor of protein synthesis in both bacterial and mammalian cells, also inhibits protein synthesis in this system. However, chloramphenicol has no affect at all on protein synthesis either in deoxycholate-treated preparations (Campbell *et al.*, 1966; Stenzel *et al.*, 1966) or in our system. Irehdiamine (Mahler *et al.*, 1966) and anhydrochlortetracycline (Suarez and Nathans, 1965) have no effect on protein synthesis in this system, and cycloheximide inhibition is not nearly as great as has been reported for other rat tissues (Trakatellis *et al.*, 1965; Bennett *et al.*, 1965). Cycloheximide inhibition was examined only at two concentrations; perhaps greater inhibition would be produced by higher concentrations. In any event, our system responds to inhibition by only two of the inhibitors tested, puromycin and cycloheximide. Inhibition appears to occur at similar levels in deoxycholate-treated and deoxycholate-free preparations. The big difference between the various systems described so far appears to rest in their ion requirements with the possible implication that these effects of ionic environment occur at the ribosome level as opposed to that of the activating and transfer system. Ca^{2+} did not appear to have any significant effect on protein synthesis and the slight inhibition which occurred at high concentrations (10 mM) may not be an effect specific to Ca^{2+} since higher concentrations of Mg^{2+} have also resulted in decreased incorporation in this as well as in other systems (Clouet *et al.*, 1966; Zomzely *et al.*, 1964).

It appears that in detergent-treated ribosomes, added Ca^{2+} at much lower concentrations results in a much more drastic inhibition (Stenzel *et al.*, 1966). Even when one-half the Mg^{2+} was replaced by Ca^{2+} in detergent-treated preparations drastic inhibition has

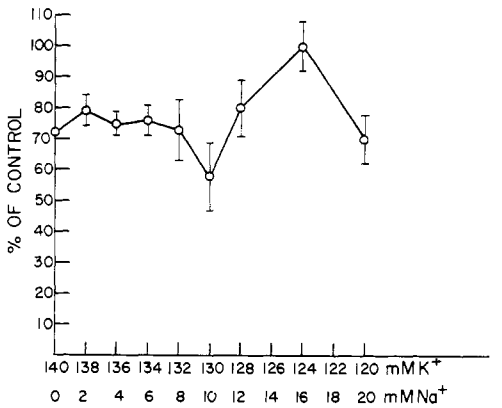

FIGURE 12: Composite of four experiments showing the effect of varying concentrations of KCl and NaCl at small increments within the range 120 mM K^+, 20 mM Na^+ to 140 mM K^+, and 0 mM Na^+. Points are expressed as per cent of the 124 mM K^+ ± standard error of the mean.

been reported (Campbell *et al.*, 1966). An interesting effect is observed when Ca^{2+} replaces Mg^{2+} with the total divalent ion concentration constant at 10 mM (Figure 9). In this case protein synthesis is actually stimulated at a mole ratio of 2 mM Ca^{2+}:8 mM Mg^{2+} but falls off rapidly as Ca^{2+} further replaces Mg^{2+}. This rapid decline in leucine incorporation is in all likelihood more a function of the inability of Ca^{2+} to replace Mg^{2+} as a divalent metal cation than a specific inhibition of protein synthesis by Ca^{2+}. It does not appear that Ca^{2+} will substitute at all for Mg^{2+} since the activity of the system with 10 mM Ca^{2+} is equivalent to that observed in the complete absence of divalent ion, Ca^{2+} or Mg^{2+}.

The effects produced by NH_4^+:Na^+ and Na^+:K^+ and NH_4^+:Na^+ are shown in Figures 10–12. For NH_4^+:K^+ and NH_4^+:Na^+ this represents the results of only one experiment for the full range of concentrations. However, four sets of concentrations were tested repeatedly for each curve with concordant results and in all probability the results are as shown: incorporation decreases as NH_4^+ ion is replaced by either Na^+ or K^+ while maintaining the total monovalent cation concentration at 140 mM. In numerous experiments the order of activity was always: 140 mM NH_4^+ > 140 mM K^+ > 140 mM Na^+. The stimulatory effects of NH_4^+ and K^+ are opposite to that observed by Clouet *et al.* (1966), while previously we (Campbell *et al.*, 1966) found no stimulation by NH_4^+. These observations are probably due to a difference in the method of preparation of the polysomes, since other preparations have been reported with an absolute requirement for K^+ for which Na^+ will not substitute (Stenzel *et al.*, 1966).

The effect of replacing K^+ by Na^+ does not appear to produce the type of curve given by NH_4^+:K^+ and NH_4^+:Na^+. At first, large 10 mM increments were chosen and the graph shown in Figure 11 was obtained. This is a composite of six experiments and in all cases the general shape of the curve was that of Figure 11. The absolute magnitude of the peaks and troughs varied (as indicated by the error bars (standard error of the means)); however, there appeared to be a definite stimulation in the region between 120 and 140 mM K^+, followed by a minimum, followed by a smaller maximum and return to the 140 mM Na^+ level. In these six experiments maximum incorporation was observed between 120 and 130 mM K^+.

Smaller increments were therefore chosen between 140 and 120 mM K^+ and the results of a number of experiments plotted in Figure 12. These results showed less variability, with a minimum appearing just before a large stimulation, the maximum occurring at precisely the same point in all four experiments. It therefore appears that in this system stimulation occurs specifically at about 124–128 mM K^+ and 16–12 mM Na^+. The absolute extent of this stimulation may very well be a function of particle-bound K^+ plus an extra increment of this ion added to the incubation medium from the medium in which the polysomes had been suspended. This amount varied from experiment to experiment since the RNP particles and pH 5 enzymes were added always on the basis of their protein content, and the volume of particle suspension added to the incubation medium, therefore, varies from preparation to preparation. This may account for 1–2.5 mM extra K^+ in the incubation tubes of various experiments. Experiments are currently underway to investigate isolation of RNP particles in media containing no Na^+ or K^+ whatever to see whether the results reported can be rendered even more reproducible.

The results reported show the utility of the present approach in studies designed to test the hypothesis of neuronal plasticity. They now permit the measurement of effects of a changing functional environment, possibly altered through continued use, on the synthetic capability of the nerve cell body. Three potential regulatory effects have been explored here: by amino acids, the Ca^{2+}:Mg^{3+} ratio, and the Na^+:K^+ ratio. In addition the very high incorporation rate *in vitro* of the polysomes described suggest their possible use as a source of functional mRNA and for the *in vitro* biosynthesis of certain proteins for which they have been preprogrammed *in vivo*.

Acknowledgments

We wish to thank Professor W. J. Moore for many stimulating discussions and Mrs. Joyce A. Wall for her fine technical assistance. Mrs. June Ewing performed the electron microscopy and Mr. Walter Vötsch and Miss Celia Weber the analytical ultracentrifuge runs.

References

Arnstein, H. R. V., and Cox, R. A. (1963), *Biochem. J. 88*, 27p.

Bennett, L. S., Ward, V. L., and Brockman, R. W. (1965), *Biochim. Biophys. Acta 103*, 478.

Bont, W. S., Rezelman, G., and Bloemendal, H. (1965), *Biochem. J. 95*, 15c.

Campbell, M. K., Mahler, H. R., Moore, W. J., and Tewari, S. (1966), *Biochemistry 5*, 1174.

Clouet, D. H., Ratner, M., and Williams, N. (1966), *Biochim. Biophys. Acta 123*, 142.

Ekholm, R., and Hyden, H. (1965), *Ultrastructure Res. 13*, 269.

Gierer, A. (1963), *J. Mol. Biol. 6*, 148.

Gray, E. G. (1964), *in* Electron Microscopic Anatomy, Kurtz, S. M., Ed., New York, N. Y., Academic, p 407.

Herriman, I. D., and Hunter, G. D. (1965), *J. Neurochem. 12*, 1937.

Korner, A. (1961), *Biochem. J. 81*, 168.

Korner, A., and Munro, A. J. (1963), *Biochem. Biophys. Res. Commun. 11*, 235.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mahler, H. R., Goutarel, R., Khuong-Huu, Q., and Truong Ho, M. (1966), *Biochemistry 5*, 2177.

Mangan, J. L., and Whittaker, V. P. (1966), *Biochem. J. 98*. 128.
Mans, R. J., and Novelli, G. D. (1961), *Arch. Biochem. Biophys. 94*, 48.
Marbaix, G., and Burny, A. (1964), *Biochem. Biophys. Res. Commun. 16*, 522.
Murthy, M. R. V., and Rappoport, D. A. (1965), *Biochem. Biophys. Acta 95*, 132.
Palade, G. E., and Siekevitz, P. (1963), *Biophys. Biochem. Cytol. 2*, 671.
Penman, S., Scherrer, K., Becker, Y., and Darnell, J. E. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 654.
Petermann, M. C. (1964), The Physical and Chemical Properties of Ribosomes, Amsterdam, Elsevier.
Pfuderer, P., Cammarano, P., Holladay, D. R., and Novelli, G. D. (1965), *Biochim. Biophys. Acta 109*, 595.
Salo, T., and Kouns, D. M. (1965), *Anal. Biochem. 13*, 74.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic.
Slater, H. S., Warner, J. R., Rich, A., and Hall, C. E. (1963), *J. Mol. Biol. 7*, 652.
Stenzel, K. H., Aronson, R. F., and Rubin, A. L. (1966), *Biochemistry 5*, 930.
Suarez, G., and Nathans, D. (1965), *Biochem. Biophys. Res. Commun. 18*, 743.
Trakatellis, A. C., Montjai, M., and Axelrod, A. E. (1965), *Biochemistry 4*, 2065.
Warner, J. R., Knopf, P. M., and Rich, A. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 122.
Webb, T. E., Blobel, G., and Potter, V. R. (1964), *Cancer Res. 24*, 1229.
Wettstein, F. O., Staehlin, T., and Noll, H. (1963), *Nature 197*, 430.
Zomzely, C. E., Roberts, S., Brown, D. M., and Provost, C. (1966), *J. Mol. Biol. 20*, 455.
Zomzely, C. E., Roberts, S., and Rappoport, D. (1964), *J. Neurochem. 11*, 567.