FACTORS INFLUENCING THE ACTIVITY OF MUSCLE PROTEIN BIOSYNTHESIS IN VITRO

by 6409

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PREFACE

I sincerely wish to express my appreciation to Dr. Gary R. Beecher, for his concerted effort and unfailing energy, devoted to this research. I am indebted to Dr. Beecher for the time he has devoted to this project, and to his guiding leadership and quest for results. I am also grateful to Dr. C. D. Hedgcoth for his advice and comments in the absence of Dr. Beecher.

I am grateful to Ernest and Ruth Ann Kiser, and to John Ruyack for their able and willing technical assistance.

I also wish to acknowledge and am grateful for the research assistantship supported by funds from the National Institutes of Health (Grant AM 13764) and for funds provided by the National Institutes of Health Biomedical Sciences Grant FR 07036 and the Kansas Agricultural Experiment Station. I wish to thank the Biochemistry department and Kansas State University for the opportunity to pursue these goals.

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INTRODUCTION

All tissues of the body are capable of synthesizing proteins, muscle being no exception. Voluminous literature exists on the isolation and characterization of macromolecular structures, control factors, and enzymes from mammalian liver necessary to support active de novo synthesis of protein in vitro. Evidence is mounting which suggests that protein synthesis in muscle may be subjected to different additional control mechanisms than those that have been elucidated for protein synthesis in mammalian liver. Primary among this evidence has been the work of Wool and coworkers, who demonstrated that in the living animal, insulin stimulated protein synthesis in muscle, but had relatively little effect on protein synthesis in liver (1). Preliminary evidence also indicated that when rats were subjected to a variety of stressful environmental conditions, including heat, cold, altitude, or cortisone injection, muscle protein synthesis in vitro was decreased, whereas liver protein synthesis in vitro was increased, compared to preparations isolated from control animals (2).

Heretofore, active in vitro muscle protein synthesis systems have utilized either partially purified amino acid activating enzymes and transfer enzymes from muscle or "cell sap" from liver in conjunction with microsomes or polyribosomes from muscle. It is pertinent that with the present knowledge and understanding of protein synthesis, that if muscle protein syn-

thesis is going to be accurately assayed *in vitro*, partial purification of enzymes from muscle or use of enzymes from liver may give an inaccurate estimate of muscle protein biosynthesis activity through the loss or addition of "control factors" from tissues other than muscle. For these reasons, the research in this thesis was oriented toward: (a) isolation of a "cell sap" preparation from muscle active in supporting protein biosynthesis in spite of the discouragement of previous authors (3), (b) isolation of a polyribosomal preparation from muscle utilizing less laborious techniques than have been reported previously, and (c) characterization of optimal conditions for the synthesis of protein using the systems isolated in "a" and "b" above.

REVIEW OF LITERATURE

The first system for muscle protein synthesis *in vitro* was reported by Florini (4) in 1962 and used a muscle microsomal system and a soluble fraction from liver for greatest activity. An expansion of that work in 1964 (5) produced optimal conditions for amino acid incorporation plus a comparision of the liver and skeletal muscle systems. The close similarities in requirements of the liver and muscle systems allowed their incubation under identical conditions. It was noted that the muscle system was similar to but far less active than analogous preparations from liver. Florini stated that the liver system was approximately 30 times more active than the system prepared from the muscle, which was attributable to the activity of the microsome fraction (4). Since the activity of amino acid incorporation was based on the microsomal RNA content (4,5,6), it was suggested that muscle microsomes were less active because they contained either less mRNA or more contaminating nonribosomal RNA than liver microsomes. Later experiments with muscle polyribosomes indicated that it was unlikely that mRNA breakdown was responsible for the lower activity of isolated muscle ribosomes. Thus, lowered incorporating activity, *in vitro*, could have been due to the presence of a membrane ATPase (7) or to an inhibitor of protein synthesis on the membrane (8).

Florini's microsomal system from muscle evolved into a system for obtaining active polyribosomes from muscle (6). The

microsomes obtained in the previous procedures, which used buffered 80 mM KCl solution, were now resuspended in buffered 555 mM KCl solution and treated with 1 % (w/v) deoxycholate (DOC) and 0.5 % (w/v) Lubrol WX[1], and the polyribosomes were sedimented through a 1.0 M sucrose cushion (6,9,10,11).

The purity of the resulting polyribosomes was relatively high, having an A_{260}/A_{280} value of 1.75 or greater and an RNA/protein value of at least 1.23. The use of low ionic strength buffers to isolate polysomes from microsomes has been shown to substantially lower the yield and activity of muscle polysomes (12).

In an attempt to overcome the laboriously long centrifugation times, and low polysomal yields and also to avoid potassium concentrations that approached those capable of disaggregating polyribosomes and ribosomes (12), Heywood et al. introduced the use of buffered 0.25 M KCl as a homogenizing medium for chick embryo muscle (13). The solubility properties of myosin (14) were utilized in those procedures to coprecipitate myosin with muscle polyribosomes at relatively low ionic strengths, thereby increasing the yield and activity of muscle polysomes (13,15). The muscle polyribosome isolation methods of Heywood et al. (13,15) have been successfully applied to rat skeletal muscle

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[1]Lubrol detergents have the generic structure: $R-(OC_2H_4)_n-OH$. Lubrol WX is the ether of cetyl alcohol ($R=C_{16}H_{33}$) where n=15-17 and has an average molecular weight of 947.

(16) and to mouse skeletal muscle (17). Chen and Young (16) added an additional centrifugation of the resuspended rat muscle polyribosomes through a 1 M sucrose cushion to increase the purity of the preparation (A_{260}/A_{280} = 1.7 - 1.8) to the values originally reported (6). Nonetheless, the latter procedures still require several long centrifugations rather than a single high speed centrifugation similar to that used for the isolation of polyribosomes from liver (19).

Heretofore, a majority of active protein systhesis systems from muscle have utilized partially purified (20-35 % and 50-65 % saturated $(NH_4)_2SO_4$ fractions) transfer enzymes in conjunction with radioactive amino-acyl-tRNA (6,9,10,11) or enzymes precipitated by adjusting the cell sap supernatant from the high speed centrifugation (muscle or liver) to pH 5 (pH 5 enzymes) in conjunction with radioactive amino acids (5,13,15,16,17). The use of aliquots of muscle or liver cell sap (105,000 xg hr supernatant) directly (4), after dialysis (15) or after Sephadex G-25 treatment (3,18) has been successful to varying degrees. The importance of various fractions from liver cell sap has been shown both indirectly (19) and directly (20) in protein synthesis systems from liver.

Incubation conditions have been optimized to varying degrees for protein synthesis systems from muscle utilizing microsomes (17) or polyribosomes (3,6,16,18,21). In general, the conditions for active protein synthesis are similar to liver

(19) and have a specific requirement for K^+, Mg^{2+}, energy and an energy regenerating system.

Microsomes and polyribosomes have routinely been isolated from muscle cells disrupted by conventional types of homogenizers (5,6,12). Dowben et al. (22) have recently demonstrated the advantage of using nitrogen cavitation to disrupt chick embryo muscle and liver cells prior to isolation of protein synthesis systems. Frazer (23) first introduced nitrogen cavitation (pressure homogenization) in 1951 but it was not until 10 years later that Hunter and Commerford (24) reported its advantages for the rupture of mammalian liver cells. Compared to rupturing cells with a Dounce tissue grinder, nitrogen cavitation gave higher yields of polyribosomes, better defined sucrose density gradient profiles and more active polyribosomes in terms of amino acid incorporation (22). I have been unsuccessful in finding results reporting the use of nitrogen cavitation to rupture muscle cells from rapidly growing or mature mammals.

EXPERIMENTAL PROCEDURE

Materials

Male Charles River rats (Charles River Breeding Laboratories, Inc., Willmington, Mass.) were used exclusively. Creatine phosphate (CP), creatine phosphokinase (CPK), L-cysteine (free base), Sephadex G-10 and G-25, and sodium lauryl sulfate (SDS) were obtained from Sigma Chemical Company (St. Louis, Mo.). Tris (base) buffer, sodium deoxycholate (DOC), bovine serum albumin (fatty acid poor), and other L-amino acids used were purchased from Schwarz/Mann (Orangeburg, New York). Dipotassium adenosine 5'-triphosphate (ATP), disodium guanosine 5'-triphosphate (GTP), and reduced glutathione (GSH) were purchased from P-L Biochemicals (Milwaukee, Wis.). Bio-Gel P-2, P-4, and P-6 were obtained from Bio-Rad Laboratories (Richmond, California). High specific activity (250 mCi/mmole) L-Leucine-^{14}C (U) was purchased from either New England Nuclear Corporation (Boston, Mass.) or Schwarz/Mann (Orangeburg, New York). Triton X-100 and 2,5-diphenyloxazole (PPO) were purchased from Packard Instruments (Downers Grove, Ill.). Lubrol WX was a generous gift of I.C.I. Organics, Inc. (Stamford, Conn.). Standard Mg^{2+} solutions (1 M) were prepared from magnesium turnings with a minimum of concentrated HCl. The concentrations were verified with atomic emission spectroscopy and the solutions were used as a source of Mg^{2+} in these experiments.

All reagents were prepared in distilled-deionized water. All other chemicals were reagent grade.

Methods

Cell sap isolation. Rats maintained on Purina Lab Chow and water ad libitum and 12 hour dark-light cycles, were sacrificed by decapitation when in the 150-250 g weight range. The rear leg muscles were removed and immediately chilled on ice. All subsequent operations were performed in a cold room (2-5°) or with pre-cooled equipment (2-5°). Excised, chilled muscle was minced with a tissue press through a screen with 1.5 mm holes. One part minced muscle was mixed into two parts (w/v) Buffer I (Table I). Glutathione was added to the buffer immediately prior to use and the pH was adjusted. Adjustment of Buffer I to pH 7.05 at 26° yields a buffer of pH 7.8 at 2°. The muscle was homogenized in buffer with a Lourdes homogenizer (Lourdes Instrument Corp., Old Bethpage, New York) using two 5-second bursts interspersed by one minute cooling in an ice bath. The homogenate was centrifuged (4°) at 13,000 xg for 15 min and the supernatant was filtered through glass wool. Six milliliters of filtered supernatant was layered directly over 7 ml of Buffer I, 0.5 M in sucrose, and centrifuged (4°) at 210,000 xg hr (Spinco 50 Ti rotor at 50,000 rpm for 55 min). This centrifugation step removed free and bound ribosomes.

The top 3 ml of cell sap supernatant, after the lipid layer had been removed, was passed through Sephadex G-10

Table I

Buffer compositions used in isolation of polyribosomes and cell sap

Buffers designated were employed as follows: Buffer I, cell sap isolation; Buffer II, equilibration of gel filtration columns; Buffer III, polyribosome isolation; and Buffer IV, resuspension of polyribosome pellet.

Buffer Number	Composition
I	100 mM Sucrose 50 mM Tris buffer, pH 7.05 at 26°C 10 mM Mg^{2+} 150 mM KCl 3 mM GSH
II	250 mM Sucrose 50 mM Tris buffer, pH 7.50 at 26°C 10 mM Mg^{2+} 80 mM KCl 3 mM GSH
III	100 mM Sucrose 50 mM Tris buffer, pH 7.05 at 26°C 15 mM Mg^{2+} 250 mM KCl
IV	50 mM Tris buffer, pH 7.50 at 26°C 10 mM Mg^{2+} 80 mM KCl

columns (other gels where indicated) to remove free amino acids and effect a buffer change. The Sephadex G-10 columns (bed size of 1.2 cm x 16 cm, except where otherwise indicated) were equilibrated with 8-10 void volumes of Buffer II (Table I). The GSH was again added to the buffer immediately prior to use and the pH was adjusted. Adjustment of Buffer II to pH 7.50 at 26° gives a buffer of pH 7.3 at 37° which is the pH at which a majority of the incorporation studies were conducted.

The protein content of the cell sap was determined by the microbiuret procedure (25). Bovine serum albumin was used as the standard for the protein determination. Buffer II was used for the dilution of cell sap to obtain the required protein levels, and also was added to the standards and blanks of the protein assay tubes.

Polyribosome isolation. Muscle was minced twice through a screen with 1.5-mm holes and then through a screen with 1.0-mm holes. One part of minced muscle was mixed into two parts (w/v) of Buffer III (Table I). Homogenization was accomplished by equilibrating the mixture with nitrogen at 900 psi (in some cases 300 psi) for 15 min in a pre-cooled nitrogen cavitation apparatus (24) and then allowing the mixture to flow through an exit valve (6.3 mm orifice, Kel-F stem; Whitey Research Tool Co., Emeryville, Calif.) and exit tube (8 mm I.D.) into a large flask. Cell disruption occurs at the valve outlet when the extra-cellular pressure is drastically lowered (to atmospheric pressure) compared to the intra-cellular pressure (at 900 psi)

resulting in the breakage of cell membranes and re-equilibration of pressures at atmospheric pressure. In some cases homogenization was conducted for two 15-sec bursts at 80 setting in a VirTis "45" homogenizer (6). The homogenate was centrifuged (4^{o}) at 13,000 xg for 15 min to sediment nuclei, mitochondria, and cell debris, and the supernatant was then filtered through glass wool.

A fresh solution of 10 % (w/v) DOC in water was prepared for each experiment. The appropriate volume was added to an aliquot of the supernatant to give a final DOC concentration of 0.05 % (w/v). The mixture was transferred to a Potter-Elvejhem homogenizer with a Teflon pestle (0.2 mm clearance) and homogenized with two strokes by hand. Twenty-five milliliters of homogenate were layered directly over 10 ml of Buffer III, 1.25 M in sucrose, and centrifuged (4^{o}) at 210,000 xg hr (Spinco type 30 rotor at 29,000 rpm for 125 min). The supernatant and sucrose cushion were carefully withdrawn by suction, the inside of the tube wiped (not disturbing the polyribosome pellet), and the tube rinsed twice with Buffer IV (Table I). The polyribosome pellet was resuspended with a Pasteur pipet in 0.3 ml of Buffer IV. Since this medium had no sucrose, it diluted the 1.25 M sucrose solution enough to allow layering of the polyribosome suspension on top of the isokinetic gradient (sucrose concentration at top was 0.465 M) used for profile analysis.

The protein content of the polyribosomal preparation was determined by the procedure of Lowry *et al.* (26). Bovine serum

albumin was used as the standard with Buffer IV added to the standards and blanks of the protein assay tubes. RNA was determined by the procedure of Fleck and Munro (27) as modified by Hallinan et al. (28). An aliquot of the polyribosome suspension (0.05 ml) was diluted with 2 ml of Buffer IV, and the appropriate absorbances determined. Ratios, A_{260}/A_{280} and A_{260}/A_{235}, were calculated as outlined by Peterman (29). Although most workers use only the A_{260}/A_{280} ratio as a criterion of polysomal purity (6,10,15,16), the A_{260}/A_{235} ratio may be more significant since 280 nm measures chiefly the aromatic amino acids and low absorption at 280 nm does not always infer the absence of extraneous protein (29).

Polyribosomal sedimentation patterns were analysed according to the procedure of Noll (30) utilizing isokinetic gradients (Buffer IV) with sucrose concentration calculated for a particle density of 1.2 g/ml. Approximately 0.2 mg of polyribosomal protein was layered on 12.4 ml gradients and centrifuged (4° sample temperature) at 400,000 xg hr (Spinco SW 41 Ti rotor at 41,000 rpm for 84 min). The gradient tube was punctured through the bottom and monitored at 260 nm while pumping (0.821 ml/min) the gradient through a micro flow cuvette (LKB Instruments Inc., Rockville, Md.) in a water-cooled jacket (4°) of a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a recorder (½"/min chart speed).

Incorporation system in vitro. The assay medium contained the following in μmoles: sucrose, 175; Tris buffer, 30; Mg^{2+}, 6; KCl, 46; ATP, 1; GTP, 0.1; CP, 20; GSH, 2.1; and 0.01 μmoles of each of 19 amino acids. The 19 amino acids (all L amino acids except glycine) used were: alanine, aspartic acid, arginine, cystine, glycine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. A solution of L-cysteine was prepared fresh and 0.01 μmoles added prior to final pH adjustment. Each assay tube contained 20 μg of CPK (20 μg will transfer 3-10 μmoles of phosphate from phosphocreatine to ADP per minute at pH 7.4 at 30°) and 0.8 μCi of L-leucine-^{14}C(U). The final pH was adjusted to 7.5 at 26°. The contents of the medium were calculated on the basis of a final reaction volume of 1 ml, whereas 0.3 ml and 0.1 ml were added by the cell sap and polyribosomal suspension, respectively. Therefore, the final medium was made up to volume to allow 0.6 ml for each 15-ml conical centrifuge (incubation) tube. The final KCl concentration was 80 μmoles with 46, 24, 8, and 2 μmoles contributed by the assay medium, cell sap, polyribosomal suspension, and ATP, respectively. The final Mg^{2+} concentration was 10 μmoles with 6, 3, and 1 μmoles contributed by the assay medium, cell sap, and polyribosomal suspension, respectively. In addition, the cell sap contributed 0.9 μmoles of GSH and 75 μmoles of sucrose, giving a final assay

concentration of 3.0 and 250 μmoles of GSH and sucrose, respectively.

The cell sap was diluted (Buffer II) to 3.45 mg protein/0.3 ml when the polyribosomal system was rate limiting or 0.855 mg protein/0.3 ml when the cell sap system was rate limiting. The polyribosomal suspension was usually diluted (Buffer IV) to 0.19 mg protein/0.1 ml for all assays. Blank tubes with Buffer IV replacing the polyribosomal suspension were run as above and used to calculate the net incorporation (CPM) by the polyribosomal system.

Each assay tube previously cooled on ice, was preincubated for 1.5 min at 37°; cell sap (0.3 ml) was added and exactly 30 sec later, the polyribosomal suspension (0.1 ml) was added and allowed to incubate for 15 min at 37°. The reaction was terminated by the addition of 1 ml of ice-cold 10 % (w/v) trichloroacetic acid (TCA) containing 0.1 mM L-leucine, to precipitate the protein. The reaction was allowed to remain overnight in an ice bath to allow complete precipitation.

The TCA precipitated protein was prepared for liquid-scintillation counting by centrifugation (4°) of the assay tubes at 1000 xg for 15 min. The pellet was resuspended, after decanting the supernatant, in 1 ml of 5 % (w/v) TCA containing 1 mM L-leucine, and heated in a boiling water bath for 15 min to hydrolyze aminoacyl residues. After cooling in ice, the tubes were centrifuged and the supernatant decanted. The pellet was subjected to three more cold TCA washes (1, 2.5, and 1 ml of

5 % (w/v) TCA, respectively) consisting of resuspension, centrifugation, and decantation. The last resuspension was performed in 0.25 ml of 5 % (w/v) SDS solution. Ten milliliters of scintillation cocktail containing toluene, methanol, and Triton X-100 in a ratio of 5:3:2 (volume ratios) with 0.3 % PPO (w/v in toluene) was used in three aliquots to transfer the solution of SDS solubilized protein from the incubation tube to a glass liquid scintillation counting vial.

The radioactivity of each sample was recorded for 10 min in a Beckman LS 200B liquid scintillation system. The channels ratio method was used to convert CPM to DPM from the appropriate quench correction curve. Results were determined and are reported on the basis of umoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein^{-1}·15 min^{-1} and umoles L-leucine-^{14}C(U) incorporated·mg polyribosomal RNA^{-1}·15 min^{-1}.

RESULTS

The level of incorporation for various cell sap protein to polyribosomal protein ratios is clearly shown in Figure 1. With the cell sap protein to polyribosomal protein ratio less than 5, the cell sap protein was rate-limiting. With an increase of cell sap protein to polyribosomal protein ratio above 8, the polyribosomal portion was rate-limiting for the system.

The activity of the cell sap in the incorporation system was effected by altering the concentrations of Mg^{2+} at various KCl levels in Buffer I during cell sap isolation (Figure 2).

Whereas the optimal Mg^{2+} concentration was 10 mM when 100 and 150 mM KCl concentrations were used, the optimal Mg^{2+} concentration increased to 15 mM Mg^{2+} at 200 mM KCl and 20 mM Mg^{2+} for 250 mM KCl. Incorporation using cell sap isolated in 200 mM KCl showed an optimum at 15 mM Mg^{2+}; results did not vary greatly in activity at other Mg^{2+} concentrations. However, incorporation using cell sap isolated in 100 and 150 mM KCl declined very rapidly at Mg^{2+} concentrations other than the optimum (10 mM). The inverse was true at 250 mM KCl concentration, where high activity was maintained at Mg^{2+} concentrations greater than 10 mM Mg^{2+} (20 mM Mg^{2+} optimum concentration).

Since gel formation was noted after the 13,000 xg centrifugation when Mg^{2+} concentrations greater than 20 mM and KCl concentrations greater than 150 mM were employed, 10 mM Mg^{2+} and 150 mM KCl were chosen as optimal concentrations in

Figure 1

Effect of varying the ratio of cell sap protein to polyribosomal protein in the assay on the amount of radioactivity incorporated. Cell sap was extracted and diluted with Buffer II to final protein concentrations of 8.5, 5.6, 4.2, 2.2, and 1.1 mg/ml so that 0.3 ml would contain 2.5, 1.7, 1.3, 0.7, and 0.3 mg of protein, respectively. Polyribosomes were diluted with Buffer IV to contain 140 µg of protein per 0.1 ml. The final cell sap protein to polyribosome protein ratios were: 18.1, 12.0, 9.1, 4.7, and 2.3, respectively.

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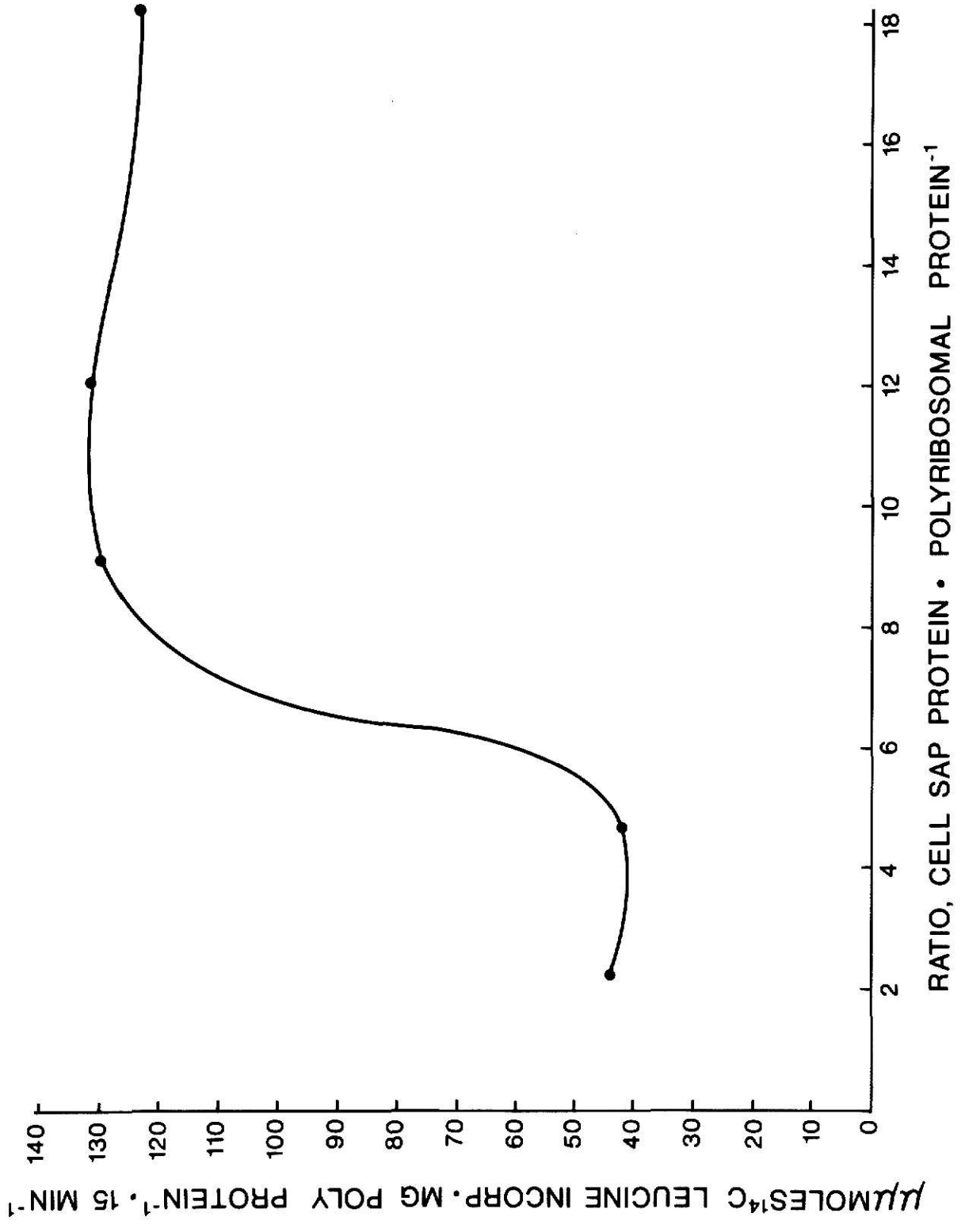
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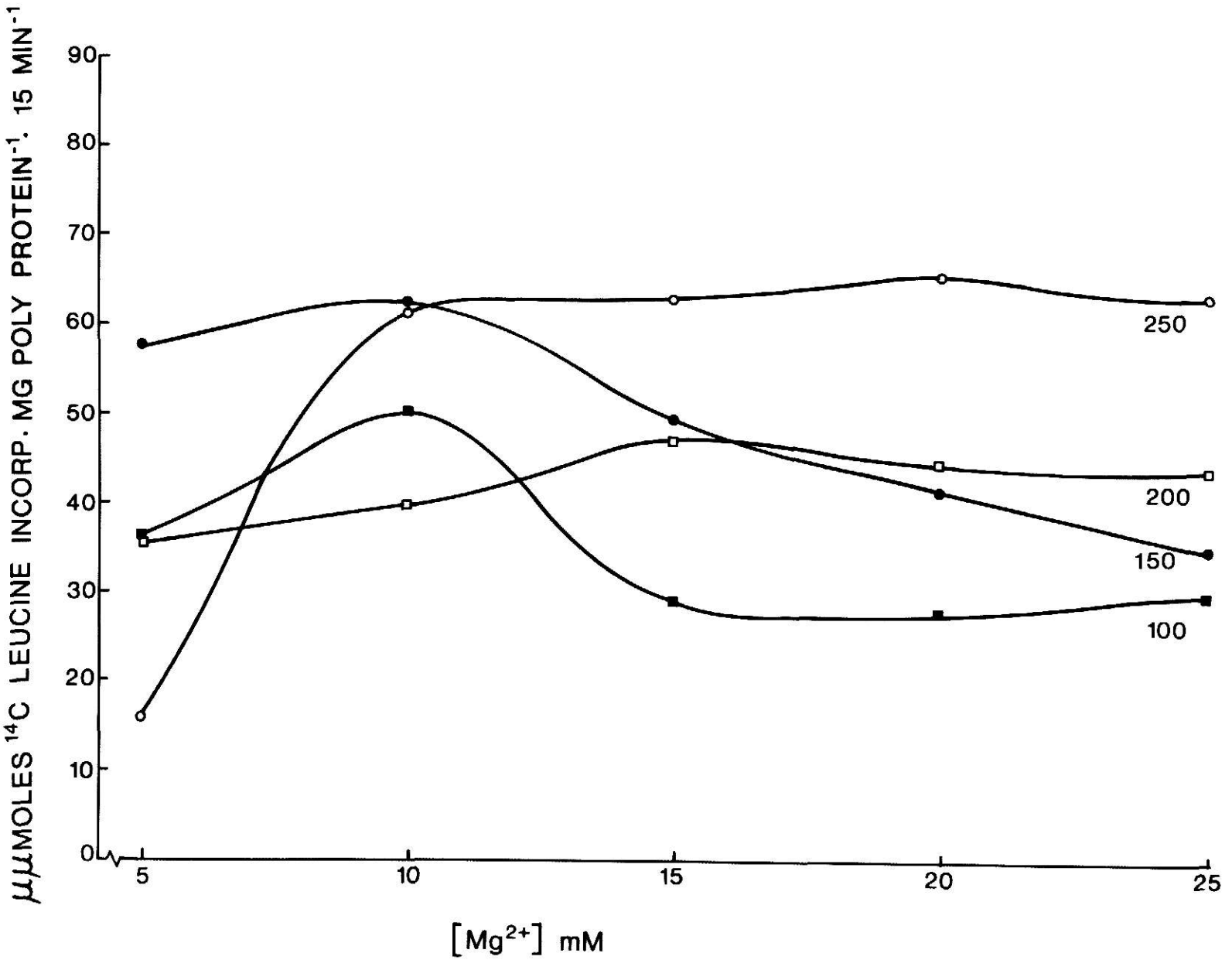
RATIO, CELL SAP PROTEIN • POLYRIBOSOMAL PROTEIN-1

Figure 2

Effect of varying Mg^{2+} concentrations at various levels of KCl in Buffer I on cell sap enzyme activity. The KCl concentration in the extraction media for cell sap were 100 (■), 150 (●), 200 (□), and 250 (○) mM. Polyribosomes were isolated in Buffer III. Assay conditions were maintained at 80 mM KCl and 10 mM Mg^{2+} with cell sap protein rate-limiting at a cell sap protein to polyribosomal protein ratio of 4.5.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the buffer for isolation of cell sap. High viscosity of muscle extracts was first noted by Szent-Gyorgyi (14) and is due to a complex of myosin and actin in a ratio of about 3:1, forming actomyosin. While the gel formation may be an intrinsic property of the cell sap at these higher ionic strengths, the mode of homogenization, using the Lourdes blade homogenizer, probably caused some difficulty, since decreasing the homogenization time and speed alleviated the difficulty slightly. Several preliminary experiments were conducted which demonstrated the superiority of the Lourdes homogenizer compared to nitrogen cavitation (900 psi) as a method of cell disruption for the subsequent isolation of an active cell sap preparation.

The optimal pH of Buffer I for homogenization and subsequent centrifugations during cell sap isolation was found to be 7.05 at 26° (Table II). Buffer I, containing Tris, at pH 7.05 (26°) gave an actual working pH of 7.8 when the buffer temperature was lowered to 2°. In practice, all buffers were adjusted to pH at 26° and then allowed to equilibrate to temperature before use. The pH of the cell sap was changed from pH 7.05 (26°) during gel filtration through Sephadex G-10 columns, which were equilibrated with Buffer II.

Addition of a sulfhydryl protectant (3 mM GSH) to the buffer for homogenizing cell sap and column equilibration yielded a cell sap preparation that was slightly more active in the assay mixture (7 % increased activity) compared to

Table II

Effect of varying pH in the buffer for cell sap isolation on amino acid incorporation

Cell sap was isolated in Buffer System I. Cell sap protein was rate-limiting during amino acid incorporation into protein (cell sap protein to polysomal protein = ratio of 4.5).

Buffer pH[a]	Incorporation[b]
6.85	36
7.05	61
7.25	50
7.55	43

[a] at 26°

[b] μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein·15 min^{-1}

buffers that were devoid of GSH. Also, addition of GSH to the buffer for cell sap isolation aided in the preservation of activity of the cell sap enzymes in the assay mixture when stored for several hours in an ice bath.

The concentration and volume of the sucrose cushion layered under the cell sap during the 210,000 xg hr centrifugation step (Spinco Type 50 Ti, 50,000 rpm for 55 min) was critical to the activity of the cell sap in the incorporation system. The exclusion of a cushion completely, compared to a cushion containing 0.5 M sucrose (normal), caused a 75 % loss of activity when the cell sap preparation was analyzed at a

rate-limiting concentration, whereas increasing the concentration of sucrose from 0.5 M to 1.25 M caused a 30 % loss of activity. The volume of the cushion was found to be important, since a change from 7 ml of a 0.5 M sucrose cushion (normal) to 4 ml of a 0.5 M sucrose cushion produced a decrease of 22 % in activity.

The centrifugal force applied to the cell sap was also demonstrated to be critical to the activity of the resulting cell sap preparation. Centrifugation for 630,000 xg hr (Spinco Type 60Ti, 60,000 rpm for 105 min) caused a 40 % loss of activity in the ability of cell sap to support amino acid incorporation into protein (assayed at a cell sap protein to polyribosomal protein ratio of 9.4) compared to cell sap prepared by centrifugation for 210,000 xg hr (normal), whereas centrifugation at 105,000 xg hr (Spinco Type 50Ti, 50,000 rpm for 28 min) resulted in a loss of 28 % activity.

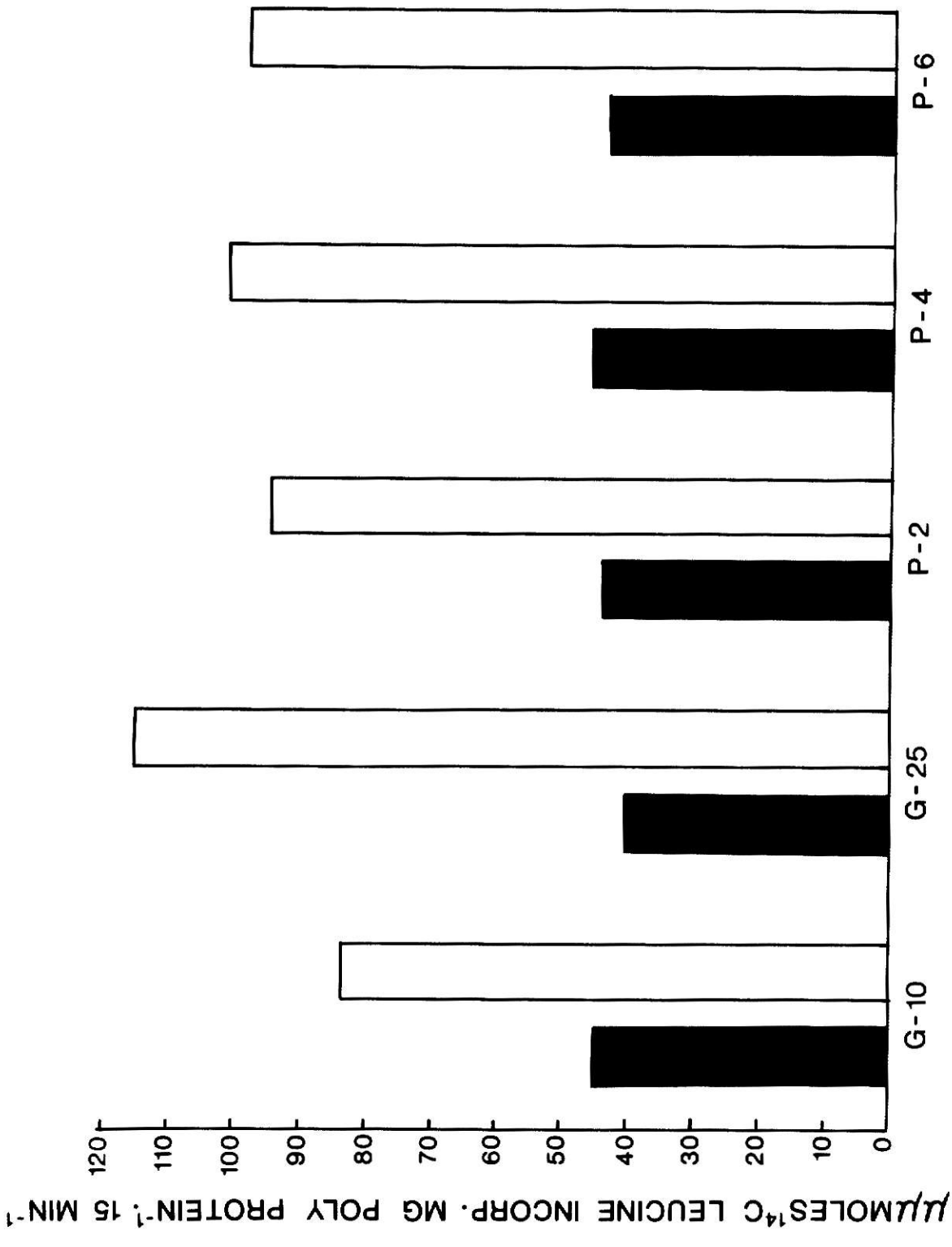
The use of gel filtration columns (Sephadex G-10) equilibrated with lower KCl (80 mM) and Mg^{2+} (10 mM) concentrations produced a cell sap preparation that had greater activity than when columns were equilibrated with higher concentrations of KCl and Mg^{2+}. This was shown when columns were equilibrated with Buffer II compared to columns equilibrated with higher concentrations of KCl (250 mM) and Mg^{2+} (15 mM) although the incorporation medium contained the same final concentrations of KCl and Mg^{2+} in each case.

Gel filtration was used effectively to remove amino acids and other small molecules from cell sap. The elution volume for the cell sap in each column size and type was determined by adding L-leucine-^{14}C(U) to the cell sap and then determining the volume of the colored cell sap that could be conveniently collected before radioactivity appeared in the eluant.

The results in Figure 3 pertain to cell sap preparations filtered through several gel filtration matrices. When cell sap was rate-limiting in the incorporation assay, no differences were observed between the gels used to filter the cell sap. Substantial differences between filtration gels were observed, however, when cell sap was in excess (polyribosomes rate-limiting) in the incorporation assay. Under these conditions, cell sap filtered through Sephadex G-25 gave the highest incorporation rates while cell sap filtered through Sephadex G-10 gave the lowest rates. The activity of cell sap preparations, assayed as above, filtered through the Bio Gel columns was intermediate between the activity extremes established by Sephadex G-10 and G-25. Experiments with cell sap filtered through Sephadex G-10 indicated that initial fractions collected from the column supported amino acid incorporation at a decreased rate compared to later fractions collected from the same column. Cell sap preparations filtered (Sephadex G-10) in small diameter columns (bed size of 1.2 cm x 16 cm) also promoted greater amino acid incorporation rates than cell sap collected

Figure 3

Effect of various filtration gels on the activity of cell sap preparations during amino acid incorporation into protein. Glass columns (bed size of 2.5 cm x 16 cm) were utilized. The five gels were equilibrated with 8-10 void volumes of fresh Buffer II prior to use. Shaded bars indicate the results obtained when cell sap was rate-limiting in the incorporation assay (cell sap protein to polyribosomal protein ratio of 4.5) and open bars are the results when polyribosomes were rate-limiting in the assay (cell sap protein to polyribosomal protein ratio of 18.8). Exclusion limits were as follows: Sephadex G-10, 700; Sephadex G-25, 5,000; Bio Gel P-2, 2,600; Bio Gel P-4, 3,600; and Bio Gel P-6, 4,600.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from larger diameter columns (bed size of 2.5 cm x 16 cm) irrespective of whether cell sap protein or polyribosomal protein was rate-limiting.

Polyribosome isolation. The criteria used for analyzing data from polyribosome experiments were a combination of results including: μμmoles of L-leucine-^{14}C(U) incorporated into protein per mg of polyribosomal protein; μμmoles of L-leucine-^{14}C(U) incorporated per mg of polyribosomal RNA; the ultraviolet absorbance ratios; and the polyribosomal density gradient profiles. It was observed that the criteria did not always correlate when isolation conditions were changed, but each criterion in itself was an aid by which to judge the quality of the polyribosomes and the environment which affected them. Polyribosomes were analyzed at cell sap protein to polyribosomal protein ratios of greater than 9, thereby defining the polyribosomes as rate-limiting in the amino acid incorporation assay (Fig. 1).

The Mg^{2+} concentration in the buffer for polyribosome isolation was optimum at 15 mM (Table III) with respect to amino acid incorporation per mg polyribosomal RNA and A_{260}/A_{280} value, whereas 20 mM Mg^{2+} gave slightly higher values when the criteria of purity were amino acid incorporation per mg polyribosomal protein and A_{260}/A_{235} values. Density gradient profiles of polyribosomes isolated in Buffer III containing 15 mM Mg^{2+} or 20 mM Mg^{2+} were similar (Fig. 4) except for a slight increase in unresolved rapidly sedimenting material in the profile of polyribosomes isolated with 20 mM Mg^{2+} buffer. Based on

Table III

Effect of varying Mg^{2+} and KCl concentrations in the polyribosome isolation buffer on polyribosomal purity and activity

Polyribosomes were isolated in Buffer III (pH 7.05 at 26°) and the purity determined by the A_{260}/A_{280} and A_{260}/A_{235} values. Polyribosomes were rate limiting in the amino acid incorporation assay.

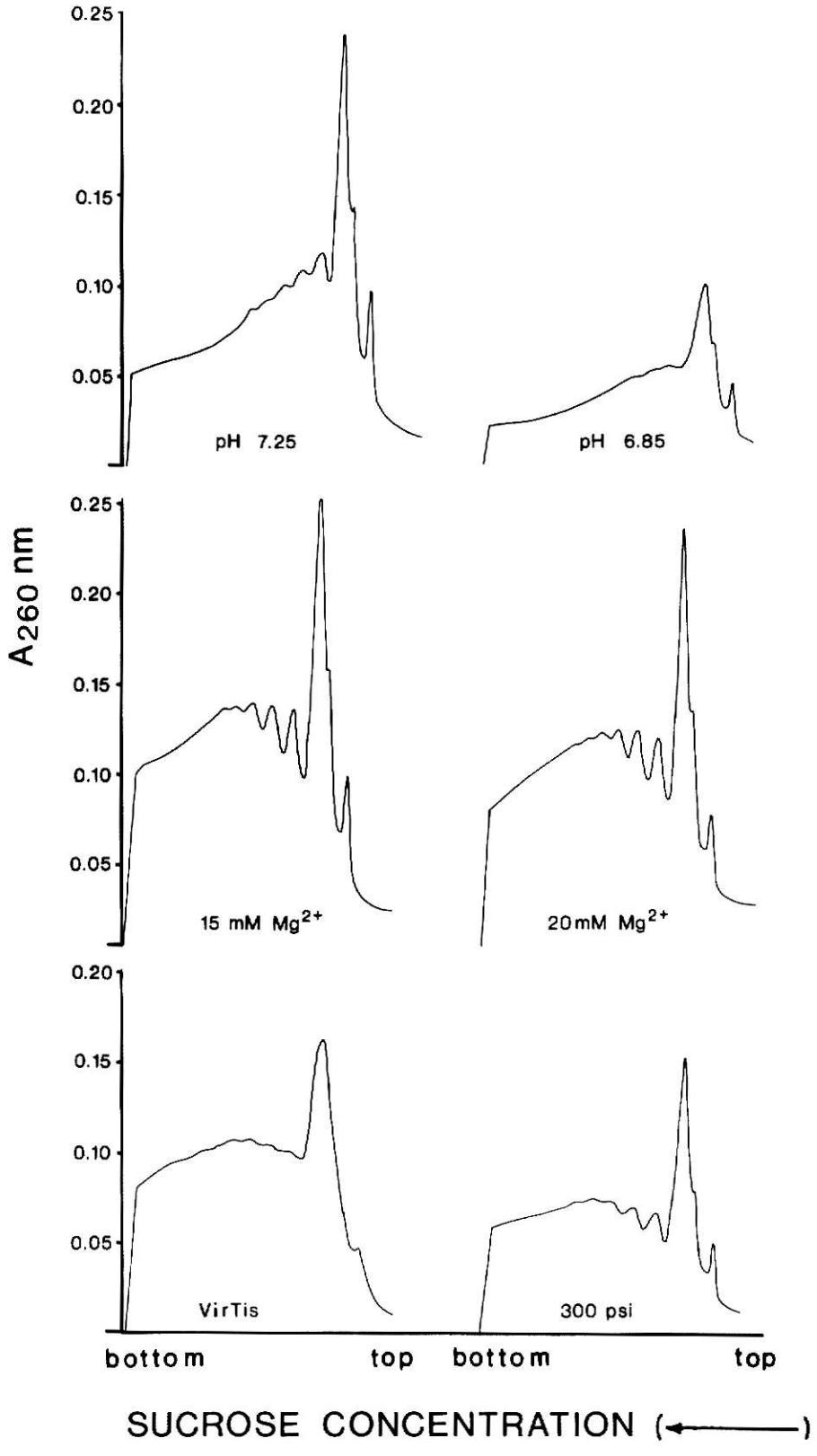
Ion mM	Incorp mg RNA[a]	Incorp mg protein[b]	$\frac{A_{260}}{A_{280}}$	$\frac{A_{260}}{A_{235}}$
Mg^{2+}				
5	77	109	1.71	1.37
10	90	92	1.72	1.39
15	115	135	1.73	1.43
20	101	138	1.56	1.44
K^{+}				
200	91	81	1.69	1.37
250	89	108	1.74	1.49
300	84	102	1.53	1.27

[a] μμmoles L-leucine-^{14}C(U) incorporated.mg polyribosomal $RNA^{-1} \cdot 15\ min^{-1}$.

[b] μμmoles L-leucine-^{14}C(U) incorporated.mg polyribosomal $protein^{-1} \cdot 15\ min^{-1}$.

Figure 4

Effects of pH and Mg^{2+} concentration in the isolation medium and mode of homogenization on the sucrose density gradient profiles of polyribosomes isolated from rat skeletal muscle. Control polyribosomes were isolated from rat skeletal muscle after cell disruption by nitrogen cavitation at 900 psi in Buffer III containing 15 mM Mg^{2+} at pH 7.05 (26^{o}). Each gradient was monitored at 260 nm with the aid of a recording spectrophotometer.


0.25
0.20
0.15
0.10
0.05
pH 7.25
pH 6.85
A260 nm
15 mM Mg2+
20mM Mg2+
VirTis
300 psi
bottom
top
bottom
top
SUCROSE CONCENTRATION (←——)

these results, 15 millimolar Mg^{2+} was determined as the optimal Mg^{2+} concentration for the isolation of polyribosomes.

The optimal concentration of KCl for the isolation of polyribosomes was 250 mM even though 200 mM KCl in Buffer III gave slightly higher activity when the results were expressed on the basis of polyribosomal RNA (Table III). The addition of 3 mM GSH to Buffer III during the polyribosome extraction was not beneficial to the amino acid incorporating activity of the system as it decreased the incorporation by 4 % on the basis of polyribosomal protein and 53 % on the basis of polyribosomal RNA.

The pH optimum of the polyribosome isolation buffer was pH 7.05 at 26° (Table IV). Sucrose gradient profiles also illustrated much better defined polyribosomal patterns and a greater proportion of rapidly sedimenting material at pH 7.05 (15 mM Mg^{2+} profile) compared to pH 7.25 or pH 6.85 (Fig. 4).

Rather surprising results were obtained when the type and concentration of detergent were altered to release polyribosomes from reticular material. When both DOC and Lubrol WX, a conventional detergent treatment in the isolation of polyribosomes from muscle microsomes (5), were used, rather respectable polyribosomes in terms of both activity and purity were isolated (Table V). However, when post-mitochondrial supernatant was treated with DOC alone, polyribosomes of high activity and purity were isolated at relatively low concen-

Table IV

Effect of varying pH in the polyribosome isolation buffer on polyribosome purity and activity

Polyribosomes were isolated in Buffer III adjusted to the pH values indicated. Polyribosomes were rate-limiting in the amino acid incorporation assay.

pH[a]	Incorp / mg RNA[b]	Incorp / mg protein[c]	$\frac{A_{260}}{A_{280}}$	$\frac{A_{260}}{A_{235}}$
6.85	38	60	1.55	1.31
7.05	75	115	1.66	1.44
7.25	71	80	1.63	1.39

[a]pH adjusted at 26°

[b]μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal RNA^{-1}·15 min^{-1}.

[c]μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein^{-1}·15 min^{-1}.

trations (0.05 and 0.10 %) of DOC (Table V). Polyribosomes of rather respectable purity and activity were isolated from post-mitochondrial supernatant which had not been treated with DOC suggesting that either a majority of polyribosomes in rat muscle are not attached to reticular material, as is the case in embryonic chick muscle (15) or that the stresses and pressures encountered during nitrogen cavitation were sufficient to release the polyribosomes from their reticular attachments. The use of detergent (DOC) concentrations greater than 1 % (w/v) resulted in a very large pellet of high protein content

Table V

Effect of various detergent treatments on polyribosome purity and activity

Various concentrations of sodium deoxycholate (DOC), alone or in conjunction with Luberol WX, were used to prepare polyribosomes from the post-mitochondrial supernatant in Buffer III. Polyribosomes were rate-limiting in the amino acid incorporation assay.

Detergent % (w/v)	Incorp / mg RNA[a]	Incorp / mg protein[b]	$\frac{A_{260}}{A_{280}}$	$\frac{A_{260}}{A_{235}}$
DOC + (1 %) Luberol WX (0.5 %)	145	129	1.64	1.26
DOC				
1.00 %	121	77	1.38	1.31
0.50	145	80	1.57	1.34
0.10	147	117	1.65	1.25
0.05	116	150	1.70	1.37
0.00	125	114	1.65	1.30

[a] μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal RNA^{-1}·15 min^{-1}.

[b] μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein^{-1}·15 min^{-1}.

during the subsequent centrifugation. These pellets had very low absorbence ratio values and were clearly not totally polyribosomal material.

Both polyribosomal amino acid incorporating activity and purity (Table VI) were affected when polyribosomes were isolated under different maximal gravitational forces. Although the total centrifugal forces applied to the polyribosomes during both isolation procedures were similar (210,000 xg hr), poly-

Table VI

Effect of varying speed of centrifugation during sedimentation on polyribosome activity and purity

Polyribosomes were sedimented at 210,000 xg hr using either a Spinco Type 30 rotor or a Spinco Type 60 Ti rotor. The Type 30 rotor ran at 29,000 rpm for 125 min (105,000 xg_{max}) and the Type 60 Ti rotor ran at 60,000 rpm for 35 min (361,000 xg_{max}). Polyribosomal protein was rate-limiting in the amino acid incorporation assay.

Maximal gravitational forces (xg_{max})	$\frac{\text{Incorp}}{\text{mg RNA}}$[a]	$\frac{\text{Incorp}}{\text{mg protein}}$[b]	$\frac{A_{260}}{A_{235}}$	$\frac{A_{260}}{A_{280}}$
105,000	165	100	1.67	1.32
361,000	128	78	1.64	1.31

[a] μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal RNA^{-1}·15 min^{-1}.

[b] μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal $protein^{-1}$·15 min^{-1}.

ribosomes sedimented under 105,000 xg_{max} forces were both more active in the amino acid incorporation assay and more pure (A_{260}/A_{280} and A_{260}/A_{235} values) than polyribosomes sedimented under 361,000 xg_{max} forces.

The concentration of sucrose in the cushion through which the polyribosomes were sedimented during centrifugation was varied in a series of experiments reported in Table VII.

Table VII

Effects of sucrose cushion concentration on polyribosome activity and purity

Polyribosomes were prepared as described in Materials and Methods and sedimented through cushions prepared in Buffer System III containing the concentrations of sucrose shown. Polyribosomal protein was rate-limiting in the amino acid incorporation assay.

Cushion Sucrose (M)	Incorp mg RNA[a]	Incorp mg protein[b]	$\frac{A_{260}}{A_{280}}$	$\frac{A_{260}}{A_{235}}$
1.35	118	78	1.72	1.41
1.25	113	98	1.74	1.49

[a] uumoles L-leucine-^{14}C(U) incorporated·mg polyribosomal RNA^{-1}·15 min^{-1}.

[b] uumoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein^{-1}·15 min^{-1}.

Results indicated that polyribosomes sedimented through 1.25 M sucrose cushions were either similar (incorporation/mg poly-

ribosomal RNA) or slightly more active (incorporation/mg polyribosomal protein) than polyribosomes sedimented through 1.35 M sucrose. Polyribosomes sedimented through 1.25 M sucrose cushions were also more pure than polyribosomes sedimented through 1.35 M sucrose.

Preliminary results showed that homogenization by nitrogen cavitation was the method of choice for the isolation of an active and pure polyribosomal preparation from muscle. Later experiments (Table VIII) indicated rather confusing results

Table VIII

Effects of homogenization conditions on polyribosome activity and purity

Muscle cells were disrupted by nitrogen cavitation at 800 psi and 300 psi or with the VirTis tissue homogenizer. Polyribosomes were isolated in Buffer III as described in Materials and Methods.

Homogenization method	$\frac{\text{Incorp}}{\text{mg RNA}}$[a]	$\frac{\text{Incorp}}{\text{mg protein}}$[b]	$\frac{A_{260}}{A_{280}}$	$\frac{A_{260}}{A_{235}}$
Nitrogen (800 psi)	70	49	1.75	1.44
Nitrogen (300 psi)	114	73	1.71	1.38
VirTis	126	108	1.70	1.41

[a] μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal RNA^{-1}·15 min^{-1}.

[b] μmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein^{-1}·15 min^{-1}.

when polyribosomes were isolated from muscle that had been disrupted by either nitrogen cavitation or the VirTis homogenizer. Amino acid incorporating activity was greatest when polyribosomes were isolated from muscle that had been disrupted by the conventional VirTis homogenizer. Nitrogen cavitation (800 psi), however, yielded polyribosomal preparations that were somewhat more pure as judged by A_{260}/A_{280} and A_{260}/A_{235} values. Sucrose density gradient profiles (Fig. 4) indicated that when polyribosomes isolated from muscle disrupted by nitrogen cavitation were analyzed (15 mM Mg^{2+} profile), profiles with a high degree of polymeric definition were obtained, whereas analysis of polyribosomes from muscle disrupted by the VirTis homogenizer showed less polymeric definition but a greater proportion of heavy molecular weight material to low molecular weight material (monosomes and disomes).

Additional data were obtained to determine the feasibility of freezing and storing active polyribosomal preparations (Table IX). A 6-8 % decrease in amino acid incorporating activity was observed when the polyribosomal material was frozen, immediately thawed, and then assayed. After 6 days of frozen storage, 57-59 % loss of activity was recorded and complete loss of activity was observed when polyribosomes were frozen (-70^{o}) and stored at relatively high temperatures (-20^{o}).

Incorporation medium. Optimal incorporation conditions were established for the polyribosomal system with polyribosomes rate-limiting (ratio of cell sap protein to polyribosomal

Table IX

Effects of freezing and storage on the ability of polyribosomes to incorporate amino acids into protein

Polyribosomal material resuspended in Buffer IV was frozen either in liquid nitrogen (-196°) or in acetone/dry ice (-70°). Samples of each freezing treatment were either thawed and assayed immediately or stored at the respective freezing temperatures for 6 days before thawing and assaying. Polyribosomes, frozen in acetone/dry ice were also stored (6 days) in a conventional freezer (-20°) before thawing and assaying.

Freezing Treatment	Storage Conditions	Incorporation[a]
Nonfrozen control		157
-196°	none	148
- 70°	none	145
-196°	6 days at -196°	65
- 70°	6 days at - 70°	68
-196°	6 days at - 20°	0

[a]umoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein^{-1}·15 min^{-1}.

protein of 18). The incorporation of amino acids into protein was determined at various times up to 60 minutes (Fig. 5). An approximately linear response was observed for the first 15 min. Subsequent to 15 min, incorporating activity began to slow and showed little activity between 30 and 60 min.

Figure 5

Effect of incubation time on the polyribosomal incorporation of amino acids into protein. Each assay tube was previously cooled in an ice bath, and allowed to preincubate for 1.5 min at 37°C, prior to incorporation.

μμMOLES ¹⁴C LEUCINE INCORP. MG POLY PROTEIN⁻¹
220
200
180
160
140
120
100
80
60
40
20
0
5
10
15
20
25
30
35
40
45
50
55
60
TIME, MIN

The effects of varying conditions in the assay medium were quite critical to the ability of polyribosomes to incorporate amino acids into protein. When Mg^{2+} concentrations deviated from 10 mM, a major loss of incorporating activity was observed (Table X). The optimal concentration of K^+ in the incubation medium was also sharply defined as 80 mM (Fig. 6). These critical concentrations of Mg^{2+} and K^+ in the incubation medium are in contrast to the somewhat broad range of Mg^{2+} and K^+ concentrations allowable in the extraction media and support the stringent ionic requirements for an active polyribosomal preparation.

Table X

Effect of varying Mg^{2+} concentration in the incubation medium on amino acid incorporation

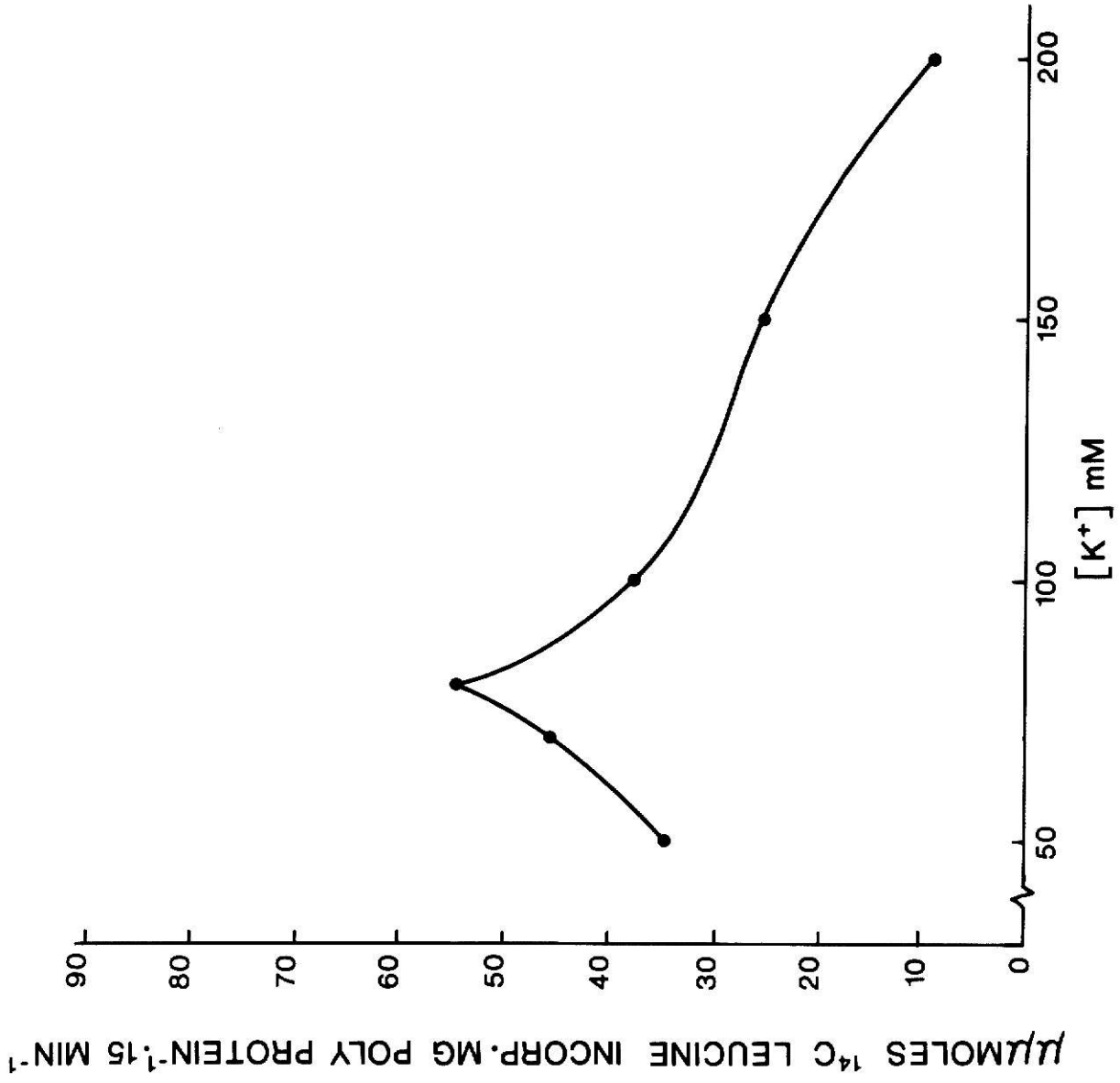
Cell sap (0.3 ml) and polyribosome (0.1 ml) contributed 3 and 1 uumoles of Mg^{2+}, respectively. Additional Mg^{2+} was added directly to the assay medium to achieve the final Mg^{2+} concentration.

Mg^{2+} mM	Incorporation[a]
7.5	56
10.0	140
12.5	64
15.0	57

[a] uumoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein^{-1}·15 min^{-1}.

Figure 6

Effect of varying K^+ concentration in the incubation medium on amino acid incorporation. Cell sap (0.3 ml), polyribosome (0.1 ml), and ATP contributed 24, 8, and 2 µµmoles of K^+, respectively. Additional KCl was added directly to the assay medium to arrive at the final K^+ concentrations shown.


μμMOLES ¹⁴C LEUCINE INCORP.MG POLY PROTEIN⁻¹.15 MIN⁻¹
0
10
20
30
40
50
60
70
80
90
50
100
150
200
[K⁺] mM

Highest amino acid incorporation rates were obtained when the pH of the incubation medium was adjusted to pH of 7.5 at 26° (Fig. 7). Tris buffer adjusted to pH 7.5 at 26° had a pH of 7.3 when the temperature of the buffer was raised to 37°. Addition of GSH to the incubation medium enhanced incorporation activity only slightly.

The polyribosomal system from muscle had absolute and specific energy requirements (Fig. 8 and Table XI). The optimal

Table XI

Effect of varying the energy system in the incubation medium on amino acid incorporation

The addition (+) or deletion (-) of components to the control incubation energy system (CP, 20 µmoles; CPK, 20 ug; and GTP, 0.1 µmoles) are indicated. Final volume of each incubation was 1.0 ml.

Addition (+) or deletion (-)	Incorporation[a]
Control	51
- 0.1 µmoles GTP	15
+ 0.1 µmoles GTP	26
+ 20 µmoles CP	26
- 20 µmoles CP	13
+ 40 µg CPK	13

[a]µµmoles L-leucine-^{14}C(U) incorporated·mg polyribosomal protein^{-1}·15 min^{-1}.

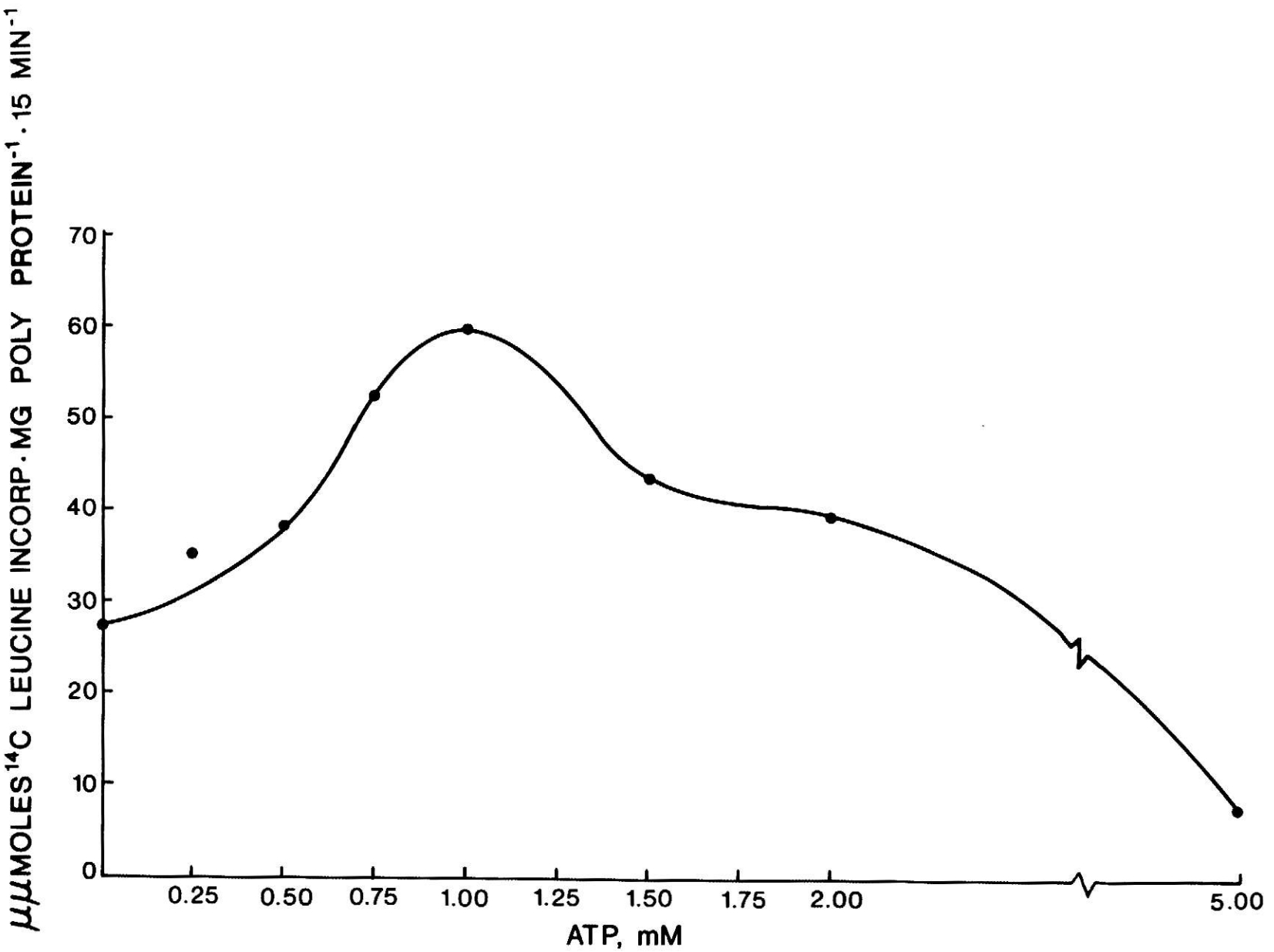
Figure 7

Effect of pH of the incubation medium on amino acid incorporation. Buffer pH was adjusted and reported at 26°.

μμMOLES ¹⁴C LEUCINE INCORP.MG POLY PROTEIN⁻¹.15 MIN⁻¹
0
10
20
30
40
50
60
70
7.3
7.4
7.5
7.6
7.7
pH

Figure 8

Effect of varying ATP concentration on amino acid incorporation into protein


μμMOLES ^{14}C LEUCINE INCORP. MG POLY PROTEIN^{-1} . 15 MIN^{-1}
70
60
50
40
30
20
10
0
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
5.00
ATP, mM

ATP concentration was 1.0 mM (Fig. 8); however, the system was approximately 50 % active when the incubation medium was devoid of ATP. Increasing ATP concentrations in the incubation system to 5.0 mM severely inhibited amino acid incorporation (Fig. 8). Table XI indicates that GTP is an essential component of the system and that 0.1 mM GTP was optimum. Removal of GTP from the incubation medium or doubling the concentration of GTP (0.2 mM) resulted in a serious loss of amino acid incorporating activity. Optimal levels of components for the ATP regenerating system were 20 mM creatine phosphate and 20 µg/ml creatine phosphokinase. Addition of creatine phosphate and creatine phosphokinase to 40 mM and 60 µg/ml, respectively, or removal of creatine phosphokinase completely, resulted in a drastic lowering (49-76 %) of amino acid incorporating activity.

DISSCUSSION

Optimal conditions for the isolation of a muscle cell sap preparation capable of supporting *in vitro* protein synthesis using muscle polyribosomes, amino acids, and an energy source have been determined. The advantages of using whole cell sap as the source of transfer enzymes, factors, and soluble RNA's are apparent when studying the entire muscle protein synthesis process *in vitro*. The interaction of tRNA with mRNA varies with tRNA prepared from different tissues (31). Precipitates of soluble enzymes from a 105,000 xg hr supernatant (pH 5.2 or ammonium sulfate fractions) may cause an unequal partitioning of these molecules between the precipitate and supernatant. In addition, several stimulation factors may be lost during such isolation procedures (20).

The use of cell sap protein levels that were rate-limiting during incorporation proved to be beneficial since optimal conditions for cell sap isolation could be ascertained. I have been able to locate only one reference in which a cell sap preparation from muscle was used to support *in vitro* muscle protein synthesis using a system similar to the system reported here (32). In those experiments, the cell sap was isolated in a buffer consisting of 8 mM Mg^{2+}, 150 mM KCl, 20 mM Tris, pH 7.6, and 10% (v/v) glycerol. In the current studies, 10 mM Mg^{2+} and 150 mM KCl were found to be optimal for

cell sap isolation in light of actomyosin gel formation during homogenization at higher ionic strength (14,33). A pH of 7.05 (26°) was found to be optimum for the isolation of the cell sap preparation. This pH is similar to the pH used by Heywood and Nwagwu (32), but drastically different from the optimum pH 6.55 at 26° for cell sap isolation from liver (19) and may reflect differences between liver and muscle tissue.

Filtering the muscle cell sap through various gels proved to be a rapid method of removing amino acids and changing buffer conditions. The presence of an effect due to the type of filtration matrix through which the cell sap was filtered when the cell sap was in excess when assayed and the absence of an effect when the cell sap was rate limiting, indicated that activators or inhibitors of protein synthesis have been included or excluded (19). The advantage of using a blade type homogenizer (Lourdes) over nitrogen cavitation to disrupt muscle cells prior to cell sap isolation suggests that nitrogen cavitation may have released membrane-bound synthesis inhibitors or the blade homogenizer may have released protein synthesis activators.

Polyribosomes having the greatest amino acid incorporating activity were isolated when Buffer III contained 250 mM KCl and 15 mM Mg^{2+}. The concentration of monovalent cation (K^{+}) has been shown to have a large affect on the yield and activity of ribosomes (13,15,16,17). Increasing concentrations of KCl up to 250 mM markedly raised the yield of ribosomal RNA (17)

whereas, concentrations above 150 mM KCl solubilized a large amount of contractile protein (12). Sufficient monovalent cation must be included in the homogenization medium to prevent binding of ribosomes and proteins to material sedimenting at 13,000 xg (13,16,34,35).

High concentrations of monovalent cations causes dissociation of polyribosomes by displacement of divalent magnesium ions which form ionic bridges between subunits (29). Martin (12) has shown dissociation of muscle ribosomes in 0.8 M KCl at 28° in the presence of Mg^{2+} ion from the 80s monomer to the 40s and 60s subunits. The subunits recombined to form active polyribosomes when excess K^+ ion was removed. Cations, such as Na^+ or K^+, compete with Mg^{2+} for sites on ribonucleoprotein (RNP) and, if present in excess, can displace Mg^{2+} but not fulfill its structural role. The RNP has been found to be more stable in the presence of K^+ than Na^+ salts (36). Liver polyribosomes isolated in buffer containing equimolar concentrations of Na^+ and K^+ reduced the amino acid incorporation activity in half (36), however heart muscle polyribosomes isolated in buffer containing 100 mM K^+ plus 50 mM Na^+ were as active and pure as those isolated in 150 mM K^+ (18). Anions which have a high affinity for Mg^{2+}, such as bicarbonate, also compete with RNP for Mg^{2+} (36). Previous studies have utilized high ionic strength buffers advantageously to solubilize myosin during homogenization with subsequent coprecipitation of polyribosomes and myosin (15,38).

The optimal pH of 7.05 (26°) in the buffer for the isolation of polyribosomes has narrow limits, similar to that in the isolation of cell sap. The pH of the homogenate must be above 7.0 to prevent binding of ribosomes to the material sedimenting at 13,000 xg (35) and to prevent precipitation of the ribosomes (29). At a higher pH of 8.0, the requirement for Mg^{2+} is greater for equivalent RNP protection against dissociation (36). The increased sensitivity to Mg^{2+} and increased solubility at higher pH suggest that electrostatic repulsion may be acting to increase the ease of dissociation (36).

Sodium deoxycholate had an affect on the amino acid incorporation ability and A_{260}/A_{280} ratios of isolated polyribosomes which supports suggestions that polyribosomes are attached to membranes (39,40). This is in contrast to chick embryo muscle (15) and cardiac muscle (18) where DOC had little affect on polyribosomal profiles and A_{260}/A_{280} ratios, respectively. Deoxycholate solubilizes microsomal membranes without apparent damage to polyribosomes as determined by their physical integrity and ability to incorporate amino acids into protein (29, 41,42). The non-ionic detergent, Lubrol WX, also removes some membrane material (29), yet DOC alone provided better conditions for the isolation of active polyribosomes for subsequent amino acid incorporation. A role for Mg^{2+} may be shown due to the dissociation of oxidized 80s ribosomes by DOC (43), whose effect may be eliminated completely by elevated Mg^{2+} concentrations (10 to 50 mM).

Profiles of muscle polyribosomes have routinely been determined in linear sucrose gradients (3,9,13,16,17). Noll (30) has described the advantages of utilizing isokinetic gradients in the determination of rat liver polyribosomal profiles. Isokinetic gradients utilized to determine muscle polyribosomal profiles (Fig. 4) indicated that the isolated polyribosomal preparations showed considerable polymeric definition and a large proportion of rapidly sedimenting material.

A method of homogenization was sought for the isolation of an active and pure polyribosome preparation from rat skeletal muscle whose homogenization was not too harsh and rigorous to yield polyribosomes that were still capable of amino acid incorporation. Bullock (44) showed that muscle polyribosomal yields were 35-50 % higher when blade homogenizers were used compared to Potter type homogenizers. It appeared that ribosomes were released with greater ease from fractions derived under more severely disruptive conditions. With the Potter type homogenizer, ribosomes became entrained with material sedimenting at slow speed.

Avis (45) studied the use of pressure homogenization, and its conditions met the criteria set forth for the isolation of active polyribosomes. He noted that under correct conditions over 90 % of the cells were ruptured with very good reproducibility of homogenates since homogenization was exempt from individual operator variations. The rupture of cells occurred

directly at the outlet valve, therefore exposing an individual cell only once to the disrupting force. Adiabatic cooling resulted from the sudden release of pressure, thereby avoiding local heating and protein oxidation was avoided by using a nitrogen atmosphere. Use of a blade homogenizer produces high shear forces which causes local heating and could damage the protein synthesizing capacity and lead to mechanical disintegration of polyribosomes. For the advantages listed, it was felt that pressure homogenization would be especially applicable to the skeletal muscle system, however the VirTis blade homogenizer was shown to be more suitable. Although Dowben et al. (22) showed enhanced amino acid incorporation with pressure homogenization over the blade homogenization, the difference between chick embryo muscle and rat skeletal muscle may be significant in response to types of cell disruption. Embryonic muscle contains a majority of single ribosomes (46) and polyribosomes which are not attached to membranes (15), whereas polyribosomes in adult tissue presumably are membrane associated. In view of the similarity of the polyribosomal profiles, the differences observed between the types of cell disruption may be subtle differences in polyribosomal tertiary structure or the release of an activator or inhibitor.

It was noted that the maximum gravitational forces applied during centrifugation of the polyribosomes through the 1.25 M sucrose cushion affected both polyribosome amino acid

incorporating activity and purity of the pellet. A change in the conformation of the polyribosome may account for the result (29).

Several investigators have determined optimal ionic conditions (5,15,21) and demonstrated the need for an energy system (3,5,6,17,21) during *in vitro* incubations utilizing components isolated from muscle. Results of the current studies indicated a similarity between ionic and energy conditions for an *in vitro* muscle protein system utilizing cell sap and conditions previously reported with partially purified fractions.

SUMMARY

An active cell sap preparation from rat skeletal muscle was isolated which supported protein synthesis in vitro and greatly enhanced the utilization of this system. Efficient isolation procedures were described with corresponding optimal conditions for amino acid incorporation into protein. The method of assay provided that either the cell sap protein or polyribosomal protein were rate-limiting in the incubation system.

Lower ionic strength buffers were more favorable for cell sap isolation providing optimal at 10 mM Mg^{2+} and 150 mM KCl, whereas the higher concentrations of 15 mM Mg^{2+} and 250 mM KCl were required for polyribosome isolation. Slightly enhanced activity was obtained using 3 mM GSH in the cell sap isolation and incubation media, but no requirement for GSH was noted in the polyribosome isolation. The pH for both the isolation of cell sap and polyribosomes was optimal at 7.05 (26^{o}), but a higher pH (7.50 at 26^{o}) was required for incubation.

Active cell sap was provided by gel filtration, using Sephadex G-10 and G-25 and Bio-Gel's P-2, P-4, and P-6. Only slight differences were noted among the filtration gels when cell sap protein was rate-limiting, whereas Sephadex G-25 gave the highest and Sephadex G-10 the lowest incorporation rates when polyribosomes were rate-limiting in the incubation system.

More active and purer polyribosomes were isolated using 0.05 and 0.10 % DOC concentrations only instead of the conventional Lubrol WX and DOC combination. The VirTis blade homogenizer provided more active polyribosomes, yet pressure homogenization yielded more pure polyribosomes and better defined sucrose gradient profiles.

The incubation medium provided optimal conditions for incorporation using 10 mM Mg^{2+} and 80 mM KCl concentrations. Incorporation of amino acids was linear for 15 min at 37^{o}. The use of an ATP regenerating system was required to eliminate high levels of addition of ATP.

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FACTORS INFLUENCING THE ACTIVITY OF MUSCLE PROTEIN BIOSYNTHESIS IN VITRO

by

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AN ABSTRACT OF A MASTER'S THESIS

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The isolation of a "cell sap" preparation from rat skeletal muscle active in supporting protein biosynthesis *in vitro* has been performed and an efficient isolation procedure which is less laborious than previous procedures was utilized. Optimal conditions were noted throughout the isolation procedures and the incorporation *in vitro* of amino acids into protein.

The optimal ionic concentrations of the buffers used to isolate cell sap preparations and polyribosomes and for the incubation system *in vitro* were: 10; 15; and 10 mM Mg^{2+}, respectively, and 150; 250; and 80 mM KCl, respectively. The pH optimum for both the isolation of cell sap and polyribosomes was 7.05 (26°), and 7.50 (26°) for the incubation system. The use of Sephedex G-25 for gel filtration of the cell sap gave the highest incorporation rates when polyribosomes were rate-limiting in the incubation system. No requirement for GSH was noted in the isolation of polyribosomes, but 3 mM GSH was beneficial in the cell sap isolation and incubation system. Low concentrations of DOC alone (0.05 and 0.10 % w/v) provided more active and purer polyribosomes than the use of greater concentrations of DOC and Lubrol WX. The VirTis blade homogenizer provided the isolation of more active polyribosomes. Pressure homogenization yielded more pure polyribosomes and better defined sucrose gradient profiles, yet lower incorporation rates than the VirTis homogenizer. Incorporation of amino acids was linear for 15 min at 37°. An ATP regenerating system was required to eliminate high levels of addition of ATP.