Amino Acid Incorporation into Protein by Cell-free Preparations from Rat Skeletal Muscle. II. Preparation and Properties of Muscle Ribosomes and Polyribosomes*

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Particulate fractions which exhibit properties generally ascribed to polyribosomes have been prepared from rat skeletal muscle. They are active in amino acid incorporation into protein; guanosine-5'-triphosphate, reduced glutathione, adenosine-5'-triphosphate, K^+, Mg^{2+}, s-RNA, and soluble enzymes are required for this process. The heavy fractions prepared by sucrose-gradient fractionation of ribosomes exhibit the highest amino acid–incorporating activity. These fractions are extremely sensitive to ribonuclease but are unaffected by deoxyribonuclease and several proteolytic and lipolytic enzymes. They are visualized in the electron microscope as extremely long chains of spherical monomers; these chains are long enough to account for the synthesis of the largest subunits of myosin. The muscle polyribosomes thus meet several criteria for the physiological significance of an isolated tissue fraction.

Two properties of skeletal muscle which lend interest to studies of protein synthesis in this tissue are its high content of one very large protein, myosin, and its sensitivity to a wide spectrum of hormones. During the characterization of the muscle–amino acid–incorporating system (a prerequisite to studies of hormone effects on muscle-protein synthesis), polyribosomes theoretically large enough to accomplish the synthesis of myosin were isolated and visualized in the electron microscope.

Analyses of rabbit muscle have demonstrated that approximately 60% of the dry weight of the tissue is composed of myosin (Szent-Gyorgyi, 1960) with a molecular weight close to 500,000 (Mueller, 1964). Myosin can be separated into subunits of approximately 200,000 mw by treatment with urea (Kielley and Harrington, 1960; Kielley and Barnett, 1961). On the basis of these observations and recent correlations between the molecular weight of proteins and the size of corresponding messenger RNA (Staehelin *et al.*, 1964; Slayter *et al.*, 1963), an assumption that muscle should contain especially large polyribosomes would be reasonable.

The properties of such polysomal structures from liver, reticulocytes, and bacteria have been studied by several groups. In general, polyribosomes are extremely sensitive to the action of ribonuclease, are the site of amino acid incorporation into protein, and are postulated to consist of chains of ribosome monomers held together by single-stranded messenger RNA (Wettstein *et al.*, 1963; Hardesty *et al.*, 1963a,b; Gilbert, 1963; Goodman and Rich, 1963; Staehelin *et al.*, 1963).

We have previously reported the preparation from rat skeletal muscle of a system in which amino acids are incorporated into protein by the classical mechanism involving s-RNA as an intermediate (Florini, 1964). Several investigators have mentioned difficulties experienced in preparing active ribosomes with high RNA-protein ratios from muscle (Wool, 1963; Earl and Korner, 1963; Florini, 1964). We have recently succeeded in isolating ribosomes from this tissue by use of a combination of several methods commonly used to prepare ribosomes from other sources (Rendi and Hultin, 1960; Jackson *et al.*, 1963). These preparations are active in amino acid incorporation and contain some polyribosomes with 100 or more ribosome monomers spaced evenly along a linear chain.

* A preliminary report of this work was presented at the meetings of the Federation of American Societies for Experimental Biology in Chicago, April 1964 (Florini and Breuer, 1964).

EXPERIMENTAL

Materials.—L-[4,5-H^3]Leucine was prepared as described previously (Florini, 1964). Cofactors and other biochemicals were purchased from the following suppliers: ATP, CTP, and GTP, Pabst Laboratories or Schwarz BioResearch, Inc.; sodium deoxycholate, Mann Laboratories: Lubrol WX, I. C. I. Organics, Inc.; GSH, California Corp. for Biochemical Research

Methods.—Microsomes were prepared from a 13,000 $\times$ *g* supernatant of rat skeletal muscle by procedures similar to those described previously (Florini, 1964) except that the initial homogenization was conducted for two 15-second bursts at maximum speed in the Vir-Tis "45" homogenizer. The microsomes were gently resuspended in a Potter-type homogenizer with a loose-fitting Teflon pestle (clearance tapered from 0.63 to 0.38 mm.). The resulting suspension was treated with 0.5 M KCl, 0.5% Lubrol WX, and 1.0% deoxycholate as described by Rendi and Hultin (1960); a systematic study of the effects of detergent concentrations on the 260/280 mμ absorbance ratio demonstrated that these conditions were optimal for the preparation of muscle ribosomes of highest purity. The mixture was then centrifuged through a 1.0 M sucrose-medium step for 2 hours at 105,000 $\times$ *g* (Jackson *et al.*, 1963) to remove the detergents and soluble proteins. The pellets were carefully removed on a spatula and resuspended in homogenizing medium by rotating in a tightly capped conical glass centrifuge tube on a vertical turntable overnight at 4°. The portion of the pellet which did not dissolve was removed by a brief centrifugation. The 260/280 mμ absorbance ratio of these ribosomes was 1.7–1.85, corresponding to an RNA-protein ratio in the range 1.2–1.5. The yield of ribosomal RNA was 100–200 μg per rat, regardless of the size of the animals; thus there was a considerable technical advantage in the use of weanling rats weighing 40–50 g.

Enzymes required for the transfer of amino acids from s-RNA to ribosomal protein were obtained by ammonium sulfate fractionation from the supernatant remaining after precipitation of the 105,000 $\times$ *g* supernatant at *p*H 5.0. Peak activities were found in the

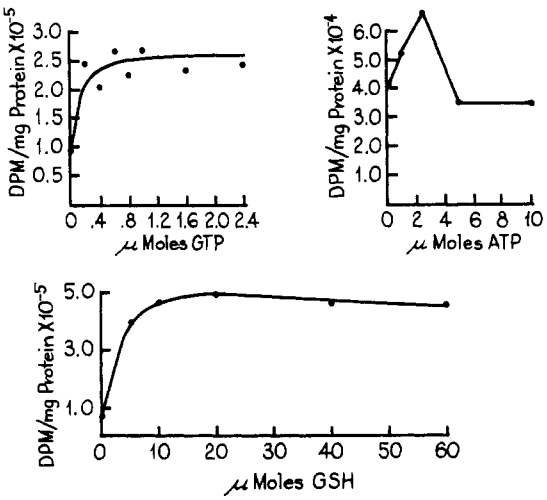

FIG. 1.—Optimal cofactor concentrations for the transfer of amino acids from leucyl-s-RNA to protein. Assay tubes contained 0.8 mg transfer-enzyme protein, 0.05–0.097 mg [^{3}H] leucyl-s-RNA, 0.15–0.25 mg ribosomal RNA, ATP, GTP, and GSH as indicated, and other components as indicated in the text.

20–35% and 50–65% saturation fractions. These were pooled, passed through a G-25 Sephadex column which had been equilibrated with homogenizing medium, and stored overnight in an ice bath.

s-RNA charged with [4,5-^{3}H]leucine was prepared from either muscle or liver *p*H 5 precipitate by the method of Bloemendal *et al.* (1962). Preparations containing specific activity of 2–10 μc/mg RNA were used routinely.

The assay for amino acid incorporation was as follows: Incubation tubes contained (in a final volume of 1.0 ml.) 2.5 μmoles ATP, 0.8 μmole GTP, 10 μmoles GSH, 90 μmoles KCl, 16 μmoles $MgCl_2$, 80 μmoles Tris-HCl, *p*H 7.5, and s-RNA with transfer enzymes and ribosomes as indicated in the legends for Figures 1 and 5. ATP and GTP were routinely prepared in equimolar magnesium. The ribosome–transfer enzyme mixture was added at zero time; incubations were conducted under air at 37° for 15 minutes. Although we previously found extensive N-terminal labeling of protein in crude muscle preparations (Florini, 1962), comparisons of the specific activity of protein hydrolysates before and after the final ether extraction of the FDNB procedure (Florini, 1964) indicated that N-terminal labeling was negligible when purified ribosomal preparations were used. The FDNB steps were thus omitted from our washing procedure in these experiments. Solvent-extracted protein was acid-hydrolyzed, dried, and dissolved in water; radioactivity and ninhydrin determinations were conducted as described in the first paper of this series (Florini, 1964).

The particle-size distribution of muscle ribosome preparations was determined by sucrose-gradient centrifugation using the method of Britten and Roberts (1960). Linear 15–30% sucrose gradients were prepared in 0.01 M $MgCl_2$, 0.08 M KCl, and 0.05 M Tris-HCl, *p*H 7.6, and centrifuged in the SW 39 rotor of the Spinco Model L ultracentrifuge for 1 hour at 35,000 rpm. Absorbancy profiles at 260 mμ were determined with a flow cell in a Beckman DB spectrophotometer attached to a Sargent SRL recorder. The gradients were removed through the top of the tube by introducing 2.0 M sucrose through the bottom at a constant rate of 0.51 ml/min, using a Harvard infusion pump. With this apparatus it was possible to stop the infusion of heavy sucrose, record the absorbance spectrum at any point

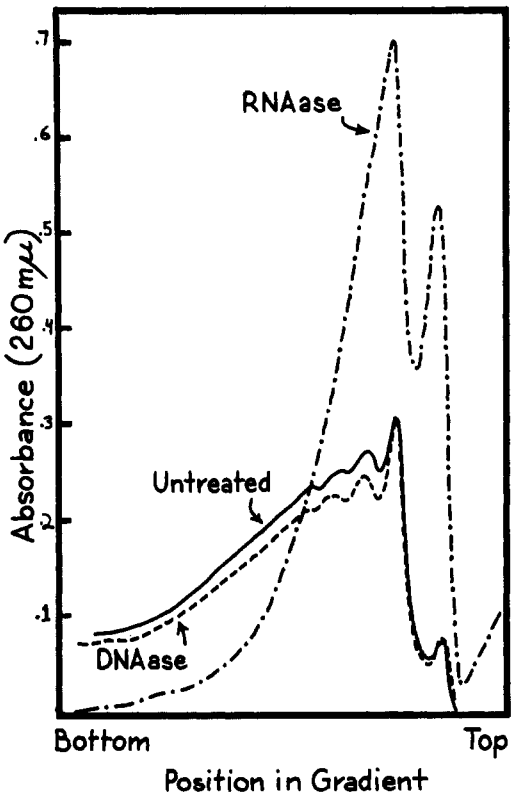

FIG. 2.—Ultraviolet-absorbance profiles after sucrose-gradient centrifugation of ribosomes prepared from skeletal muscle of 50-g rats. Ribosomes (0.3 mg RNA) suspended in homogenizing medium were layered on linear 15–30% sucrose gradients and centrifuged under conditions described in the text. Nuclease treatments were by the method of Gilbert (1963). Similar effects of nucleases were observed when detergent-treated microsomes were incubated with the enzymes and added directly to gradients without prior pelleting.

in the gradient, and resume scanning of the tube contents.

For electron microscopy droplets of ribosome samples were placed on Formvar-coated grids and excess fluid was removed by touching filter paper to the edge of the grid. The grids were then washed several times with buffer-salt solution to remove sucrose. The grids were shadowed with Pt-Pd and photographed in an RCA EMU-3C electron microscope at magnifications of 8,000–14,000.

RNA content of ribosomes was routinely calculated from the 260 mμ absorbance using an extinction coefficient of 20 cm^2/mg. Protein content of transfer-enzyme preparations was determined by the biuret procedure of Gornall *et al.* (1949). Amino acid concentration in protein hydrolysates was determined by the ninhydrin procedure of Yemm and Cocking (1955).

RESULTS

Characteristics of the Transfer Reaction.—The cofactor requirements for the transfer of amino acids from s-RNA into protein are shown in Figure 1. The ATP requirement was assayed in the presence of 0.8 μmole/ml of GTP, and the GTP optimum was found in the presence of 2.5 μmoles/ml of added ATP. An ATP-regenerating system was not required with these purified tissue fractions. Optimal concentrations of KCl and $MgCl_2$ for incorporation were 100 and 15 μmoles/ml, respectively. Incorporation was linear under optimal conditions for 60 minutes; approximately 20% of the added label was transferred from s-RNA to

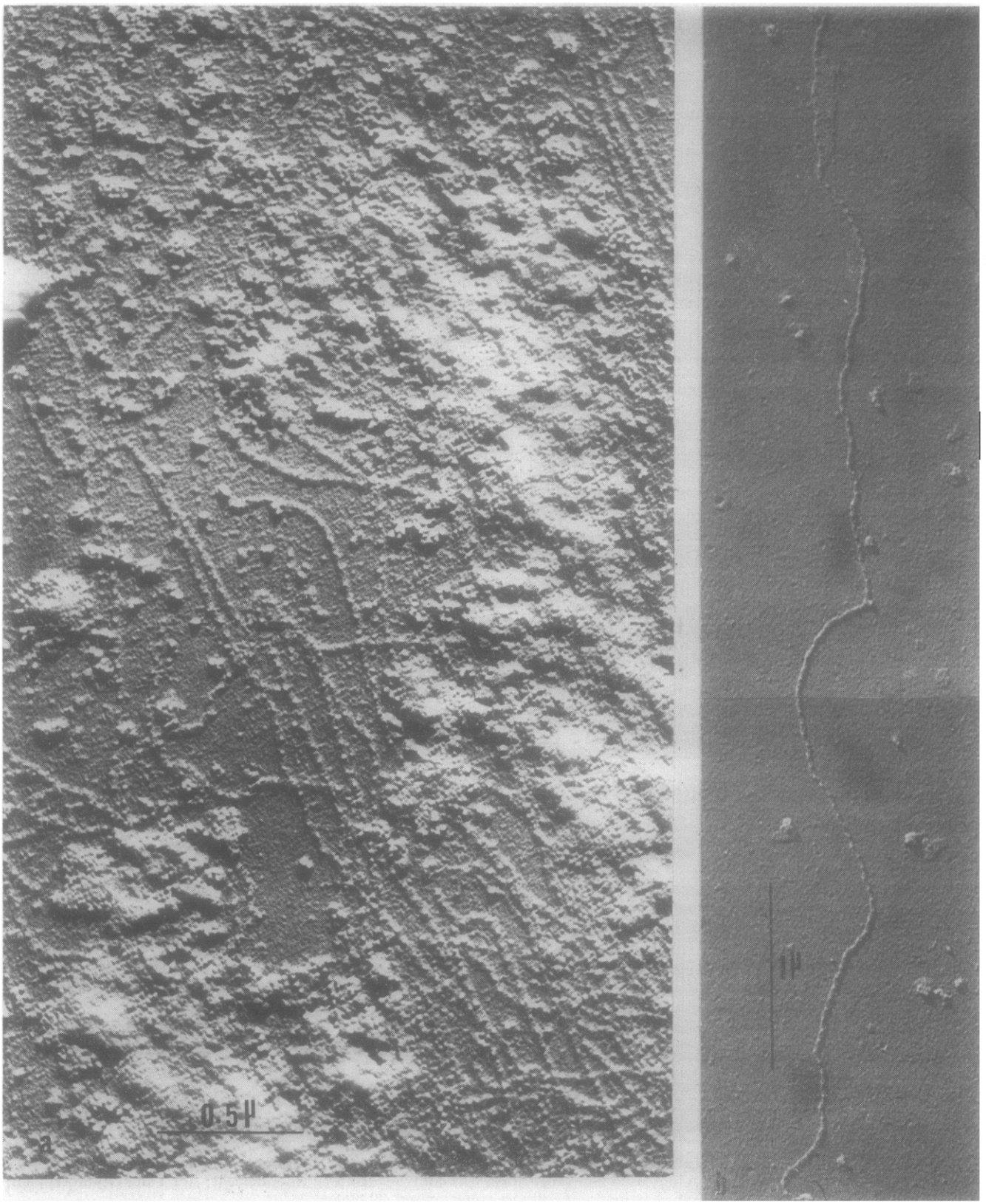

FIG. 3.—Electron micrographs of rat skeletal muscle–ribosome preparations. (a) Concentrated field taken from resuspended pellet of total ribosomes, 38,000×; (b) isolated chain of polyribosomes from a sucrose-gradient fractionation, 25,500×. This chain was found in a fraction indicated by the arrow in Figure 5. Preparation of ribosomes for electron microscopy was as described under Methods.

protein. The transfer reaction was inhibited 85% by the addition of 10^{-4} M puromycin. Transfer of [^{14}C]-phenylalanine from either liver or muscle s-RNA was stimulated by the addition of polyuridylic acid. Similar requirements and optima were observed for the incorporation of free [4,5-^{3}H]leucine into protein by the muscle ribosomes; of course, the *p*H 5 precipitate and an ATP-regenerating system were also required.

Sucrose-Gradient Centrifugal Analyses of Ribosomes.—Sucrose-gradient centrifugation of muscle ribosomes

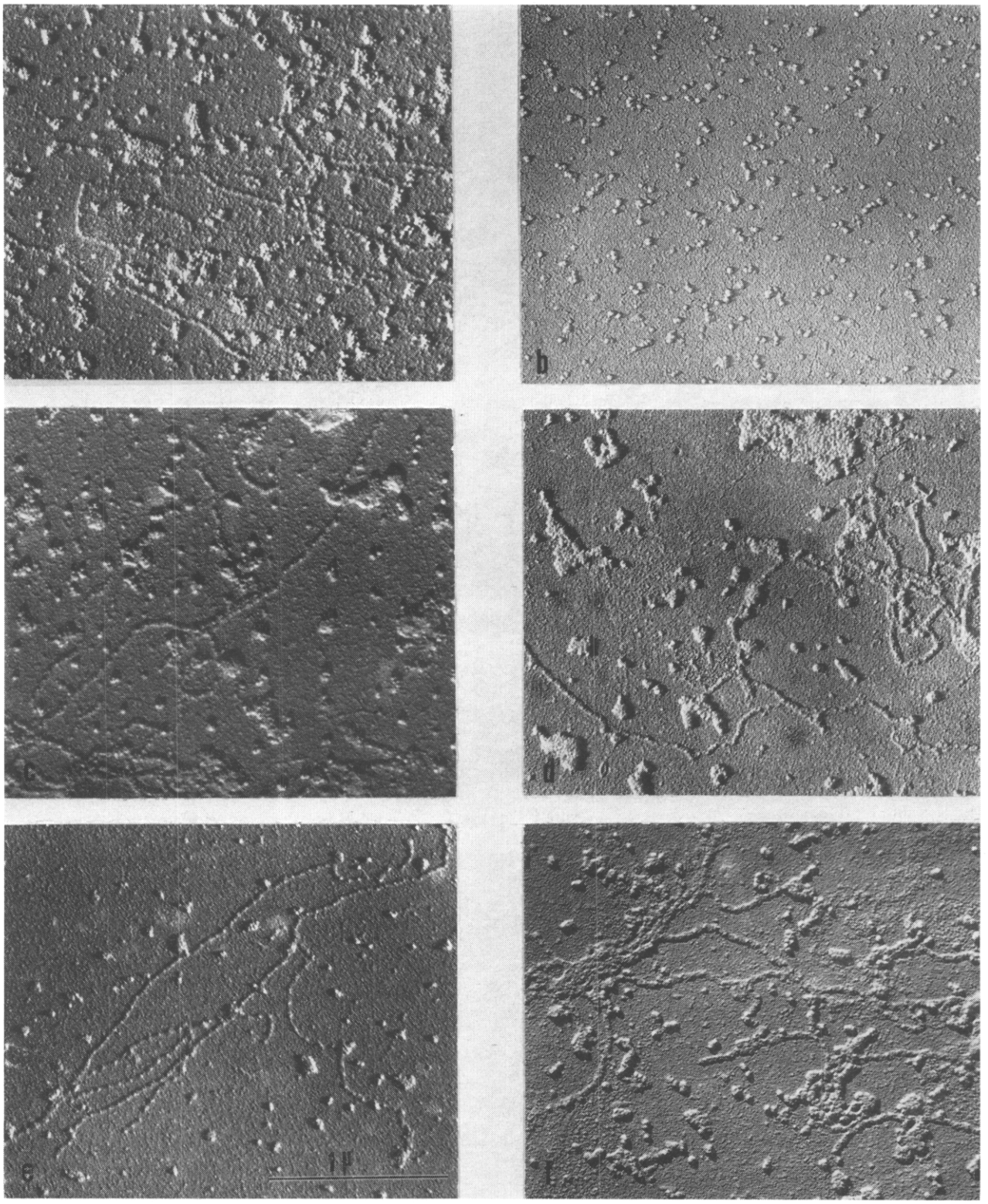

FIG. 4.—The effects of enzyme treatments on the appearance of skeletal muscle ribosomes. All enzymes were incubated with ribosomes at 4° for 1 hour at the indicated concentrations; (a) control; (b) 5 μg ribonuclease; (c) 1 mg chymotrypsin; (d) 100 μg deoxyribonuclease; (e) 200 μg papain; (f) 200 μg collagenase; 22,300×.

revealed the presence of material sedimenting much more rapidly than ribosome monomers, as shown in Figure 2. Ultraviolet-absorption spectra typical for ribonucleoprotein (Takanami, 1962) were observed throughout the gradient. The marked sensitivity to ribonuclease and insensitivity to deoxyribonuclease of the heavy material suggested strongly that it consisted of polyribosomes, i.e., of ribosomes held together by a chain of messenger RNA. In order to test this assumption further, muscle ribosomes were incubated for 1 hour at 4° with the following enzymes: papain, collagenase, chymotrypsin, lysozyme, and wheat germ lipase. There was no discernible effect on the sucrose-gradient profiles or disruption of the chains as visualized in the electron microscope (see Fig. 4). Incubation of these enzymes with their natural substrates under similar conditions showed that they were active. For example, papain was assayed by the method of Kunitz

(1935) using denatured hemoglobin as substrate; 88% of the substrate was hydrolyzed after 1 hour at 4°. Collagenase was tested with azocoll hide powder as substrate by the procedure of Warrack *et al.* (1946) with detectable activity seen after 1 hour in the cold. These two enzymes were also tested for contaminating RNAase activity by the method of Kunitz (1946); none was found. Deoxyribonuclease was assayed by the method of Kunitz (1950).

Electron Microscopy of Muscle Ribosomes.—Examination of muscle ribosomes in the electron microscope gave results in agreement with those of the sucrose gradients. Figure 3a shows that the muscle preparations included many strikingly long chains of ribosomes; we have found chains containing more than 100 monomers. The concentration of ribosomes in this field is too great to allow separation of individual chains; it is presented to indicate the frequency with which these chains occur in our preparations. Figure 3b presents a micrograph of the longest polyribosome of which we have a clear individual picture. This was taken from a sucrose gradient at the position indicated by the arrow in Figure 5. Similar structures have been found throughout the lower portion of sucrose gradients and were also seen when detergent-treated microsomes were added directly to gradients without prior pelleting through concentrated sucrose. The structure in Figure 3b contains approximately 115 monomers 250 A in diameter on a chain of total length approximately 72,000 A. The distance between monomer centers is roughly 600 A, which is consistent with the suggestion made by Staehelin *et al.* (1964) that the monomers are separated by about 90 nucleotides, i.e., approximately 7 A per nucleotide. The monomer size and spacing has been consistent in all muscle-ribosome preparations we have examined.

Figure 4 shows the effects of treatment with various enzymes on the appearance of the ribosome preparations in the electron microscope. In some cases, the relatively high concentration of enzyme protein (0.2–1.0 mg/ml) caused a loss of clarity in the micrographs, as in the case of collagenase. Chymotrypsin, on the other hand, appears to have removed extraneous material from the spaces between the ribosome monomers. It is clear, however, that the only enzyme which had any effect on the length or frequency of occurrence of the long chains was ribonuclease; this enzyme was added at a concentration of only 5 μg/ml. Under these conditions we were unable to find any chains.

Activity of Sucrose-Gradient Fractions.—Fractions collected in the cold from sucrose gradients of muscle ribosome preparations were assayed for activity in the transfer of amino acids from s-RNA to protein. The results are presented in Figure 5, which is a composite of duplicate determinations. It may be seen that the greatest specific activity is found in the polyribosome region of the gradients with minimum activity in the monomer area. These data are similar to those reported for liver, reticulocyte, and bacterial systems.

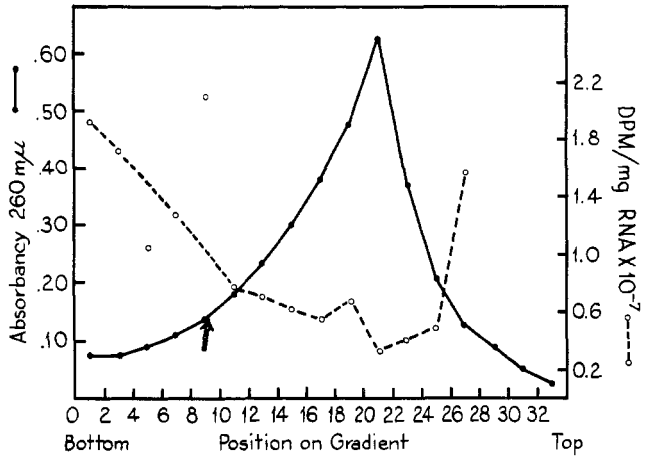

FIG. 5.—Amino acid-incorporating activity of sucrose-gradient fractions of muscle ribosomes. Fractions (0.3 ml) were mixed with 0.5 ml of solution containing 0.8 μmole GTP, 2.5 μmoles ATP, and 10 μmoles GSH in homogenizing medium at *p*H 7.6, and [^{3}H] leucyl-s-RNA containing 5.54 × 10^6 dpm. Incubations and isolation of protein for liquid scintillation counting were as described in the text.

DISCUSSION

Ribosomes which were active in protein labeling from aminoacyl-s-RNA or free amino acids have been prepared from rat skeletal muscle by a combination of well-established techniques. Although the yield of ribosomes from muscle was small, requirements for amino acid incorporation into protein were typical of similar preparations from other sources. The muscle ribosomes, *p*H 5 enzymes, and transfer enzymes were completely interchangeable with analogous fractions from liver; only the liver ribosomes showed a greater specific activity.[1] This might be taken to mean that the protein synthetic machinery is identical in all cell types within an animal. It would be genetically economical to have only two genes for ribosomal RNA, two for transfer enzymes, and fifty or sixty for s-RNA, rather than a complete set for each tissue. The preparations of ribosomes from rat heart (Earl and Korner, 1963; Zak *et al.*, 1964; Rampersad and Wool, 1964), brain (Acs *et al.*, 1961), and prostate (Liao and Williams-Ashman, 1962) as well as liver and skeletal muscle will allow more extended studies on the interchangeability of the tissue fractions involved in protein synthesis.

Probably the most interesting feature of the muscle ribosomes is their structure as visualized in the electron microscope. The length and linear structure of these polyribosomes are apparently unique to muscle;[2] possibly these long structures have survived the isolation procedure because of a relatively low nuclease content in muscle. It would be anticipated that the very long chains are fragile to shear forces generated during the isolation procedure, and that muscle is involved in the synthesis of proteins of various sizes; accordingly, we find a wide distribution of chain lengths in electron micrographs. The shorter polyribosomes are seen as clusters similar to those which have been found in other preparations (Staehelin *et al.*, 1963; Warner *et al.*, 1962). However, chains are consistently found in our preparations bearing 60–100 monomers, and some are even longer.

Chains containing this many monomers might be expected to sediment very rapidly, whereas we find these structures in portions of sucrose gradients which would contain material with sedimentation constants of the order of 300–400 S (Martin and Ames, 1961). On the basis of molecular weight, polyribosomes containing only 15–20 ribosomes would be expected to exhibit such sedimentation characteristics. However, this correlation of particle weight and sedimentation characteristics is based on the properties of ideal spherical molecules; the extremely long, linear chains visualized in the electron microscope would sediment less

[1] J. R. Florini and C. B. Breuer, unpublished observations.

[2] While this manuscript was in preparation, Kretsinger *et al.* (1964) reported the preparation from chick embryo of polyribosomes containing at least 100 ribosomes, as estimated from the sedimentation coefficients.

rapidly than spheres of the same mass at these concentrations.

The enzyme sensitivities of the long structures provide compelling evidence that they are held together by RNA rather than by DNA, protein, or lipid. It is also significant that the greatest amino acid–incorporating activity was found in those sucrose-gradient fractions which contained the large polyribosomes. Thus it seems reasonable to conclude that these structures are indeed polyribosomes and not protein artifacts of the isolation procedure.

Evidence from two groups indicates that these large polyribosomes are found in, as well as isolated from, muscle. Waddington and Perry (1963) found similar structures *in situ* in muscle in developing frog embryos, and Cedergren and Harary (1964) found structures resembling long polyribosomes in electron micrographs of sections through beating-heart cells in culture. Both groups interpreted the structures as ribosomal aggregates. We are not aware of the existence in, or isolation from, muscle of any subcellular component other than polyribosomes which would have the same spectrum of enzyme sensitivities, be of the same size, and be isolated by the same procedures as the long structures found in our ribosome preparations. For example, although the myosin aggregates prepared by Mueller *et al.* (1964) and by Rice (1961) show a general resemblance to the muscle polyribosomes, the globular ends of the myosin molecules are only one-sixth as large as the ribosome monomers. Furthermore, myosin would presumably be sensitive to papain and insensitive to ribonuclease.

It is interesting to estimate the size of a protein which could be assembled on a polyribosome such as that shown in Figure 3b; this calculation can be made on the basis of several alternative assumptions. If the messenger RNA is fully extended, as is suggested by the spacing between ribosome monomers, then the length of each triplet would be 21 A (Slayter *et al.*, 1963), the chain would contain 3400 triplets, and the molecular weight of the protein product would be approximately 340,000. Martin (1963) has presented evidence for the existence in *Salmonella typhimurium* of messenger RNA of this length (molecular weight of 4×10^6). Similarly, if chain length is estimated from the number of ribosome monomers it contains, and this length is correlated with the size of the reticulocyte ribosomal aggregate (Slayter *et al.*, 1963), the polyribosome in Figure 3b could produce a protein with a molecular weight of 350,000. Alternatively, if the messenger RNA in the polyribosome of Figure 3b is tightly coiled, then the displacement per triplet would be only 10.5 A, and the protein product could have a molecular weight approaching 700,000.

Kiho and Rich (1964) have suggested that β-galactosidase is assembled on the polyribosome from twelve small subunits. Kielley and Harrington (1960) have shown that concentrated urea or guanidine hydrochloride causes dissociation of myosin into subunits of mw 200,000; these are the smallest subunits of myosin obtained by nonproteolytic processes. Messenger RNA large enough to form a polyribosome containing 60–70 ribosomes would be required to synthesize a protein of this size; as Figures 3 and 4 show, our preparations contain many structures in this size range.

The muscle polyribosomes thus meet three criteria for a physiologically significant role in muscle protein synthesis: They are active in amino acid incorporation under conditions typical for protein synthesis in other systems, similar structures have been observed in intact muscle, and they are large enough to account for the synthesis of the largest subunits of myosin.

Note Added in Proof

Heusson-Stiennon (1964) has recently published an elegant electron microscopic study of the synthesis of myofibrils in embryonic rat muscle. She concluded that the myofibrillar proteins are synthesized on large polyribosomes. This is additional evidence for the presence and function in muscle of the polyribosomes we have isolated and described in this paper.

Acknowledgments

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Formamidine Sulfinic Acid as a Biochemical Reducing Agent

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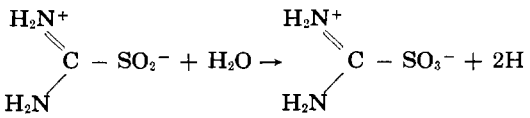
Formamidine sulfinic acid (thiourea dioxide) is described as a useful biochemical reducing agent for converting substrates such as ferredoxin, cytochrome *c*, methemoglobin, and the like, to their reduced forms. The reagent can be used in aqueous buffered systems (*p*H 4–8), and has the advantages of producing no by-products which can interfere with spectroscopic measurements. The chemical and spectroscopic properties of the compound are described.

Frequently, in biochemical reactions involving electron transport, a chemical reagent is required to convert an oxidized molecule to the reduced state at mild reaction conditions. The two widely used reagents for this purpose are sodium borohydride and sodium dithionite. These have certain disadvantages, however, which prevent their application in spectroscopic studies; sodium dithionite produces colloidal sulfur with its inherent light-scattering properties, and sodium borohydride evolves hydrogen gas which interferes with absorption measurements. In the course of experimental work with cytochrome *c* (Shashoua, 1964), we observed that formamidine sulfinic acid (thiourea dioxide) can readily reduce the oxidized ferric molecule to the reduced ferrous state at room temperature in aqueous solutions without the above-mentioned disadvantages. This feature and a number of other properties recommend the use of thiourea dioxide as a general biochemical reducing agent.

Properties of Formamidine Sulfinic Acid

Formamidine sulfinic acid is known as a useful reducing agent for both inorganic and organic compounds, such as tin and copper salts in ammoniacal solutions (Böeseken, 1936), and certain quinones and dyes (Gore, 1954). The mechanism of its reducing action is reputed (Gore, 1954) to involve an irreversible conversion to formamidine sulfonic acid in accordance with the reaction scheme:



Recent evidence (Golunyad and Bolovtova, 1962) has suggested that its reducing action at 70° involves further decomposition to give sulfur dioxide as a by-product. However, in using an aqueous medium and a maximum reaction temperature of 40°, no indication of such a problem was detected. A study of the redox potential of formamidine sulfinic acid as a function of concentration and *p*H gave a value of −1.5 volts for the *p*H range 4–8 at concentration levels of 4×10^{-4}–1×10^{-2} M.

One major advantage of formamidine sulfinic acid as a biochemical reducing agent is that both the reduced and oxidized (sulfonic acid) forms of the compound are water soluble and optically transparent above 300 mμ.

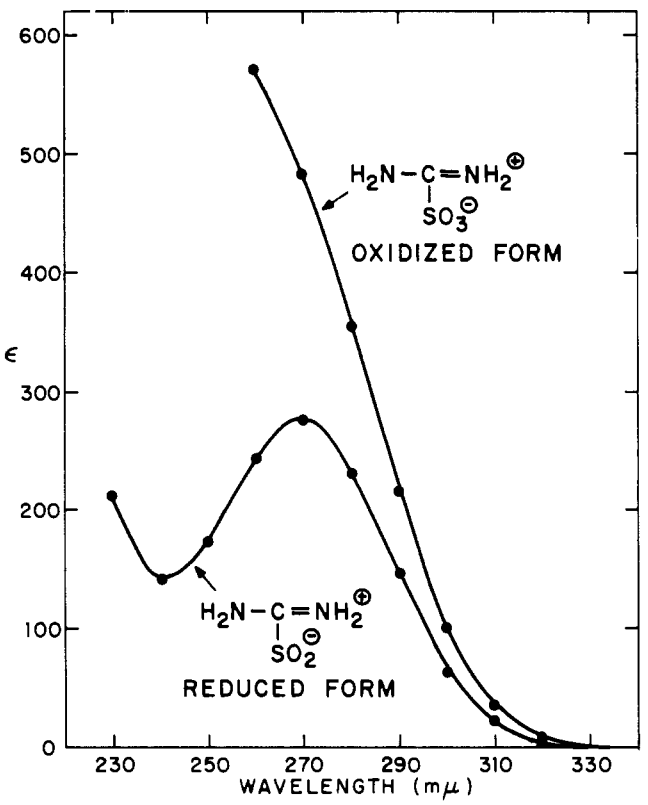


FIG. 1.—Absorption spectra of aqueous solution of formamidine sulfonic acid (oxidized form) and formamidine sulfinic acid (reduced form).

Figure 1 shows a plot of the absorption spectra of the reduced and oxidized forms. (The oxidized form, formamidine sulfonic acid, was prepared by reaction with excess hydrogen peroxide.) One disavdantage of the compound is perhaps its slow reaction rate of 4–16 hours in a typical experiment.

Experimental Results and Discussion

Oxidized cytochrome *c*, methemoglobin, ferredoxin, peroxidase, vitamin B_{12}, and ferritin are readily converted by formamidine sulfinic acid to their reduced forms. A typical reaction consists of treating a 0.02% solution of the oxidized substrate at *p*H 7 with about 50–200 molar equivalents of formamidine sulfinic acid