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Amino Acid Incorporation into Protein by Cell-free Preparations from Rat Skeletal Muscle. III. Comparisons of Activity of Muscle and Liver Ribosomes

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ABSTRACT: Comparisons of amino acid incorporation into protein by liver versus muscle ribosomes have been made under conditions in which ribosomes were limiting. Liver ribosome were two to three times as active as muscle ribosomes; addition of synthetic messenger ribonucleic acid (polyuridylic acid) abolished this difference. Similar observations were made when either liver or muscle was the source of soluble RNA, transfer enzymes, and *p*H 5 precipitate, and when either free amino acid or amino-acyl-s-RNA was the labeled precursor. There was no evidence that endogenous inhibitors lowered the activity of the muscle preparation. On the basis of these and earlier results, it is concluded that the large differences in protein labeling by liver and muscle *in vivo* and *in vitro* are attributable both to the lower ribosome content of muscle and to the lower activity of the isolated muscle ribosomes which is probably a result of their lower content of m-RNA.

Our primary interest in studying amino acid incorporation into protein by rat skeletal muscle preparations *in vitro* has been the elucidation of mechanisms controlling protein synthesis in this tissue. The previous papers in this series (Florini, 1962; Florini, 1964; Breuer *et al.*, 1964) presented characterizations of the muscle system at various levels of purification. In general, the muscle system was similar to but far less active than analogous preparations from liver, even when activity was expressed on the basis of the RNA content of the microsome fractions. We have suggested (Florini, 1964) that muscle microsomes were less active because they contained either less messenger RNA or more contaminating nonribosomal RNA. This paper presents evidence which supports the former suggestion, although the possibility remains that entrapped cytoplasmic RNA was partially responsible for the lower activity of the muscle microsomes when incorporation was expressed in terms of RNA content of the microsomes.

Experimental

Materials and Methods. The materials, techniques, and procedures used in this study were described in detail in paper II in this series (Breuer *et al.*, 1964). Ribosomes of high purity (260/280 mμ absorbancy ratios of 1.75 to 1.83) were prepared from muscle or liver of 18-hour starved normal male rats (Carworth Farms CFN strain) by treating the resuspended microsomal pellet with 0.5% Lubrol WX and 1.0% deoxycholate followed by centrifugation through a layer of 1.0 M sucrose. The 20–35% and 50–65% saturated $(NH_4)_2SO_4$ fractions of the *p*H 5-soluble portion of the 105,000 $\times$ *g* supernatant were combined, passed through Sephadex G-25, and used as the transfer enzyme preparation. s-RNA was charged with the specified labeled amino acid by the method of Bloemendal *et al.* (1962). Contents of incubation tubes are specified in the legends to the tables. After incubation for 15 minutes at 37°, samples were precipitated with 5% trichloroacetic acid, washed by the Siekevitz (1952) procedure, and hydrolyzed, and the specific radioactivity of the resulting amino acid solution was determined. Results are expressed as $\mu\mu$moles of amino acid incorporated or transferred into protein, although it is apparent that appreciable dilution of the very highly labeled amino acids occurred when the tracer was added to the relatively large quantities of amino-acyl-s-RNA present in the incubation mixture (Table I) or used for the charging of unstripped s-RNA. Thus the observed labeling represents the lower limit of amino acid incorporation actually occurring; it is probably several orders of magnitude lower than the

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Received October 5, 1964.

actual incorporation. This consideration also precludes comparisons of absolute incorporation between experiments in which different quantities of *p*H 5 fraction were used.

TABLE I: Incorporation of Labeled Amino Acid into Protein.[a]

Expt.	Ribosomes mg RNA	Origin	μμMoles Amino Acid Incorporated/mg Ribosomal RNA: [4,5-^{3}H]-Leucine	[U-^{14}C]-Phenylalanine No Poly-U	[U-^{14}C]-Phenylalanine +200 μg Poly-U
1	0.125	Muscle	4.8		
	0.125	Liver	12.8		
	Ratio, liver/muscle		2.66		
2	0.335	Muscle		13.8	194
	0.268	Muscle		13.5	159
	0.201	Muscle		6.3	120
	0.134	Muscle		8.4	121
	Average, Muscle			10.5	149
	0.476	Liver		23.4	124
	0.381	Liver		18.1	115
	0.286	Liver		19.9	147
	0.190	Liver		20.0	141
	Average, Liver			20.4	132
	Ratio, liver/muscle			1.94	0.89

[a] Incubation tubes contained (in μmoles): ATP, 1; creatine phosphate, 20; GTP, 0.3; $MgCl_2$, 10; KCl, 80; Tris-HCl, *p*H 7.6, 50; sucrose, 250; and 20 unlabeled amino acids, 10^{-3} M each (omitted when poly-U added); in a final volume of 1.0 ml. In addition, tubes of experiment 1 contained 1.25 mμmoles (20 μc ^{3}H) L-[4,5-^{3}H]leucine, *p*H 5 precipitate (0.6 mg protein) and transfer enzymes (1.7 mg) from 7.8 g muscle (added with muscle ribosomes), or *p*H 5 precipitate (0.6 mg) and transfer enzymes (1.7 mg) from 3.6 g liver (added with liver ribosomes). Tubes of experiment 2 contained 28.4 mμmoles (0.4 μc ^{14}C) L-[U-^{14}C]phenylalanine, *p*H 5 precipitate (1.1 mg protein) from a mixture of 0.9 g muscle and 2.7 g liver, soluble fraction (0.35 mg) (passed through Sephadex G-25) from 0.01 g each of liver and muscle, and the amount of ribosomes expressed as their content of RNA (determined from the 260 mμ absorbancy using an extinction coefficient of 20 cm^2/mg). Samples were incubated and prepared for counting as described previously (Breuer *et al.*, 1964).

Results

Incorporation of Amino Acids into Protein. Table I presents comparisons of free amino acid incorporation into protein by ribosomes from liver and muscle. In experiment 1, *p*H 5 precipitate and transfer enzymes

TABLE II: Transfer of Labeled Amino Acids from s-RNA to Protein.[a]

Expt.	Ribosomes mg RNA	Origin	μμMoles Amino Acid Transferred/mg Ribosomal RNA: [4,5-^{3}H]-Leucyl-s-RNA	[U-^{14}C]-Phenylalanyl-s-RNA No Poly-U	[U-^{14}C]-Phenylalanyl-s-RNA +200 μg Poly-U
1	0.28	Muscle	2.89	0.378	
	0.57	Muscle	2.94	0.345	
	0.85	Muscle	3.01	0.345	
	Average, Muscle		2.95	0.356	
	0.28	Liver	9.42	0.575	
	0.57	Liver	10.07	0.764	
	0.85	Liver	7.41	0.672	
	Average, Liver		8.97	0.670	
	Ratio, liver/muscle		3.04	1.88	
	0.28 each	Liver + Muscle	3.56	0.302	
	Calculated	Liver + Muscle	3.45	0.267	
2	0.17	Muscle		1.25	6.88
	0.18	Liver		2.67	6.67
	Ratio, liver/muscle			2.14	1.03

[a] Incubation tubes contained (in μmoles): ATP, 2.5; GTP, 0.8; GSH, 10; KCl, 90; $MgCl_2$, 16; sucrose, 250; and Tris-HCl, *p*H 7.6, 80; in a final volume of 1.0 ml. In addition, to the tubes of experiment 1 were added: 0.1 mg ^{3}[H]leucyl-s-RNA containing 29 μμmoles (0.46 μc ^{3}H) L-[4,5-^{3}H]leucine or 0.1 mg ^{14}[C]phenylalanyl-s-RNA containing 7.1 μμmoles (0.001 μc ^{14}C) L-[U-^{14}C]phenylalanine and 1.0 mg of transfer enzymes prepared by $(NH_4)_2SO_4$ fractionation of a 1:1 mixture of liver and muscle 105,000 × *g* supernatant. To the tubes of experiment 2 were added: 0.18 mg ^{14}C-phenylalanyl-s-RNA containing 30.2 μμmoles (0.0043 μc ^{14}C) L-[U-^{14}C]phenylalanine and 0.5 mg of transfer enzymes prepared from the same tissue as the ribosomes. Ribosome concentrations were determined as described in Table I.

from the same tissue were incubated with the ribosomes; in experiment 2 (and in other experiments not reported here), a mixture of liver and muscle *p*H 5 precipitates and transfer enzymes were used with ribosomes from both tissues; this did not significantly affect the ratio of incorporation by liver and muscle ribosomes. (This ratio has been consistently greater in experiments with labeled leucine than with labeled phenylalanine; this may be explained by the assumption that the ratio of leucine to phenylalanine is higher in the liver proteins than in the muscle proteins labeled under these conditions.) The difference in phenylalanine incorporation by the muscle and liver preparations completely disappeared when saturating amounts of poly-U were added to the incubation mixture.

Transfer of Amino Acids from s-RNA into Protein. Table II presents the results of experiments in which amino-acyl-s-RNA was used as the source of labeled amino acids. (In the experiments reported here, liver s-RNA was used for convenience; essentially identical results were obtained in less extensive experiments using muscle s-RNA.) It should be noted that large excesses of s-RNA were added to ensure that ribosomes would be limiting; hence the per cent of the labeled amino acid transferred from s-RNA into protein was relatively low.

When equal quantities of muscle and liver ribosomes were incubated together (experiment 1), incorporation was essentially additive. The difference in phenylalanine incorporation by muscle and liver ribosomes was essentially eliminated by addition of poly-U. Thus the results with the transfer reaction directly parallel those obtained when incorporation of free amino acids was being measured.

Comparisons of Other Liver and Muscle Fractions. No differences in the activities or specificities of *p*H 5 precipitates, transfer enzyme fractions, or isolated s-RNA prepared from either liver or muscle could be observed by our techniques. In all cases, the yield per gram of tissue was somewhat greater from liver than from muscle. However, compartmentalization occurs to a striking extent in muscle; a very large portion of the muscle mass is composed of myofibrils which may have a very limited permeability to the soluble components of the cell (Walls, 1960). Indeed, because of the very low concentration of ribosomes in skeletal muscle (Breuer *et al.*, 1964), it seems likely that the ratio of activating enzymes, s-RNA, and transfer enzymes to ribosomes may be greater in this tissue than in liver, and that soluble components do not limit the incorporation of amino acids into muscle protein.

Discussion

Twenty years ago it was shown that the turnover of muscle proteins is appreciably slower than that of liver proteins (Shemin and Rittenberg, 1944). More recently, McLean *et al.* (1958) have demonstrated that incorporation of injected amino acids into protein of the microsome fraction occurs more slowly in muscle than in liver. We have previously reported (Florini, 1962, 1964) similar differences in the activity *in vitro* of subcellular preparations from muscle and liver at two levels of purification. On the basis of these observations, our experience in the preparation of ribosomes from muscle (Breuer *et al.* 1964), and the data presented in this paper, we conclude that the relatively slow synthesis of protein in intact muscle can be attributed to two factors: (1) muscle contains far less ribosomes than does liver,[1] and (2) isolated muscle ribosomes contain substantially less messenger RNA (m-RNA) than similar liver preparations.[1] We have found no evidence that the activity of muscle preparations might be limited by the presence of inhibitors in muscle soluble fractions, microsomes, or isolated ribosomes.

The properties of these tissues and the techniques required for ribosome isolation must be considered in evaluating the foregoing conclusions. Analyses of the RNA content of liver and muscle by Devi *et al.* (1963) indicate that approximately eight times as much RNA is found in liver. About 80% of the liver RNA is associated with ribosomes (Hoagland, 1960); thus muscle must contain far less ribosomal RNA than liver. How much less it contains is difficult to estimate because of uncertainties in the subcellular distribution of muscle RNA (Margrath and Novello, 1964). Thus, although much of the muscle RNA sediments with the myofibrils (Perry and Zydowo, 1959), under some conditions an appreciable portion can be found in the sarcotubular (i.e., microsomal) and soluble fractions (Muscatello *et al.*, 1961).[2] It seems likely that the distribution of muscle RNA is to some extent dependent on the procedure used to homogenize the muscle. Porter and Palade (1957) have shown that the endoplasmic reticulum of rat muscle contains relatively few ribonucleoprotein particles when examined by electron microscopy. From all these lines of evidence, it is clear that the ribosome content of muscle is substantially lower than that of liver, although the absolute difference is not accurately known. We usually isolate fifteen to twenty times as much ribosomal RNA per gram of tissue from liver as from muscle of rats weighing 100–150 g.[1]

A more serious complication is the possibility that our techniques might damage muscle m-RNA more than liver m-RNA—i.e., the difference in activity of isolated ribosomes (which we have attributed to a corresponding difference in m-RNA content) might be an artifact of the isolation procedure. This question could be answered by a direct determination of the amount of functioning m-RNA in muscle and liver, but an unambiguous direct comparison of m-RNA

[1] Both the ribosome and apparent m-RNA content of muscle vary to some extent with the age of the animals (C. B. Breuer and J. R. Florini, unpublished results), but they remain significantly below the liver content of these fractions.

[2] We regret having failed to cite this prior report of amino acid incorporation *in vitro* by a muscle preparation in our earlier papers and are grateful to Dr. William Scher for calling it to our attention.

content of muscle is not technically feasible at present. Short-term *in vivo* labeling of RNA would measure rate of synthesis rather than total content of m-RNA and would be complicated by differences in isotope dilution in the two tissues. Direct extraction of the total m-RNA would presumably measure nuclear as well as cytoplasmic m-RNA; such an extraction of microsomes or ribosomes would involve the same uncertainties as measurement of amino acid incorporation. Thus our determination of m-RNA by its functional activity (as enzymes are determined by their activities) seems the most satisfactory approach. The low ribonuclease content of muscle (Roth and Milstein, 1952) and the relatively high stability of the large polyribosomes isolated from muscle (Breuer *et al.*, 1964) indicate that it is unlikely that m-RNA breakdown is responsible for the lower activity of isolated muscle ribosomes.

It might be argued that the experimental design was biased so that only differences in activity of the ribosome fraction would be observed. However, intermixing experiments (Florini, 1962, 1964) as well as the yields of s-RNA, *p*H 5 precipitate, and transfer enzymes prepared from muscle indicate that soluble components were present in very large excess in the crude muscle homogenates or upon addition of *p*H 5 precipitate to isolated microsomes; very large decreases in the activities of the soluble components could occur before any effect on amino acid incorporation would be detected.

It was to be expected that the activity of mixtures of muscle and liver ribosomes would be additive; we have previously reported (Florini, 1964) that addition of muscle microsomes or *p*H 5 precipitate did not inhibit incorporation by liver microsomes, and we concluded that the muscle preparations did not contain inhibitors which could account for the lower activity of muscle ribosomes. It should be noted that our earlier results with microsomes are not in conflict with the recent report by Hoagland *et al.* (1964) of an inhibitory factor in microsomes from normal liver. Our experiments were conducted with a large excess of liver microsomes; the contribution of the muscle microsomes to the total incorporation was negligible, and inhibition by the liver microsomes would not have been detected. Furthermore, GTP was added in all our determinations, so the effect of Hoagland's inhibitor would have been minimized.

Three kinds of quantitative control of the activity of isolated microsomes or ribosomes have now been described: (1) negative control via general inhibition of protein synthesis as suggested by Hoagland *et al.* (1964), (2) inherent variation of ribosome activity independent of m-RNA content (Lucas *et al.*, 1964), and (3) control of m-RNA synthesis or availability to the ribosomes. This last mechanism occurs in bacteria (Jacob and Monod, 1961) and evidence is rapidly accumulating that it occurs in mammalian cells as well. Hiatt and associates (Henshaw *et al.*, 1963; DiGirolamo *et al.*, 1964), in studies on rat liver preparations, have obtained evidence that ribosomes not associated with m-RNA are not active in amino acid incorporation into protein. The observation by many workers (Maxwell, 1962; Weinstein and Schechter, 1962; Gardner *et al.*, 1962; Fessenden *et al.*, 1963), in addition to this report, that liver ribosomes are stimulated by addition of poly-U suggests that the activity of the liver preparations, like those from muscle, is limited by their content of m-RNA. That m-RNA content controls the activity of other mammalian tissues has been concluded from a number of studies on the effects of various hormones on specific target tissues (Schmidt, 1964). Liao and Williams-Ashman (1962) used the addition of synthetic m-RNA (as we have in Table II) to demonstrate that the effect of testosterone administration on valine incorporation was attributable to effects on the m-RNA content of ribosomes isolated from the ventral prostate. Experiments now in progress in this laboratory[3] suggest that testosterone administration has similar effects on the m-RNA content of ribosomes isolated from skeletal muscle.

Acknowledgments

We are grateful to Dr. P. H. Bell for advice, interest, and encouragement throughout this study, and to J. D. Patterson for skil ful technical assistance.

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Preparation of Mannobiose, Mannotriose, and a New Mannotetraose from *Saccharomyces cerevisiae* Mannan

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ABSTRACT: The deacetylated products of an acetolysate of yeast mannan have been separated on a Sephadex column to yield pure di-, tri-, and tetrasaccharides in high yield. The structure of each was established by methylation and periodate oxidation, as well as by comparison with authentic compounds. The substances isolated were the disaccharide *O*-α-D-mannopyranosyl-(1→2)-D-mannopyranose, the trisaccharide *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannopyranose, and a tetrasaccharide *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannopyranose. The tetrasaccharide cannot be fitted as a unit into the most recent structure proposed for yeast mannan, and this suggests that alternative structures must be considered for this polysaccharide.

Yeast mannan, one of the main soluble polysaccharides of yeast cell wall, can be obtained easily by autoclaving whole yeast cells with dilute alkali or neutral buffer. Because bakers' yeast, *Saccharomyces cerevisiae*, is so readily available, most structural studies have been carried out on mannan extracted from this microorganism.

Several investigators have studied the mannan structure by methylation; and, in general, the results are consistent despite the different methods of preparation (Table I). It is clear from these studies that yeast mannan is highly branched and contains 1,2′-, 1,3′-, and 1,6′- linkages. On the basis of methylation data, Haworth *et al.* (1941) proposed several possible structures, the fundamental feature being an α-1,2′-linked backbone with branch points involving α-1,6′- linkages.

Recently, however, Peat *et al.* (1961b) have isolated a series of α-1,6′-linked oligomannosides by acid hydrolysis of yeast mannan, and, in conjunction with methylation work (Peat *et al.*, 1961a), they proposed a structure in which an α-1,6′-linked chain forms the backbone of the polysaccharide, the 1,2′- linkages being at branch points.

TABLE I: Methylation Analysis of Yeast Mannan.

O-Methyl-mannose	Molar Ratios Reference		
	Haworth *et al.* (1941)	Cifonelli and Smith (1955)	Peat *et al.* (1961a)
2,3,4,6-Tetra-	2	2	15
3,4,6-Tri-	1	1	7
2,4,6-Tri-	1	1	7
2,3,4-Tri-	Trace	Trace	1
3,4-Di-	2	2	14

In what began as a routine study, we undertook the acetolysis of yeast mannan to prepare authentic mannose oligosaccharides to be used as proton magnetic resonance standards in the determination of the chemi-

* From the Department of Biochemistry, University of California, Berkeley. *Received October 14, 1964.*